Small GTP-binding proteins in the regulation of exocytosis in mast cells

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ABSTRACT

Permeabilised rat peritoneal mast cells can be stimulated to secrete by the addition of calcium and GTPyS. If, however, the cells are stimulated subsequent to permeabilisation, they become increasingly refractory to stimulation, being only able to secrete, for example, 30-40% of their contained hexosaminidase after 10 minutes compared to almost 100% when stimulated at the time of permeabilisation. This phenomenon is called “rundown” and is thought to be due to the loss of cytosolic proteins involved in exocytotic regulation which leak from the cell through the membrane lesions created by the permeabilising agent.

Using the recovery from rundown as the basis of a bioassay, I have collaborated on the purification of two such cytosolic factors from bovine brain cytosol which appear to act as exocytotic regulators. One factor was fully purified and identified as a complex of Rac1 (a small GTP-binding protein of the Rho family) and Rho GDI. I found that the purified complex retards rundown, whilst Rho GDI accelerates rundown when applied alone.

Rac2 is understood to be the predominant form of Rac expressed in myeloid cells. I have purified recombinant Rac2 from E.Coli and found that it retards rundown when preactivated by binding GTPγS before application to the cells, confirming that Rac can play a role in mast cell secretion. In addition, I found that a dominant negative mutant form of Rac2 inhibits secretion induced by GTPγS.

Cdc42, another Rho-related GTPase which can interact with some Rac effectors, was also purified from E.Coli. Preactivated Cdc42 was found to retard rundown, but the concentration-effect relationship indicates that Cdc42 interacts with more than one effector. Also, a dominant negative mutant Cdc42 inhibits GTPγS-induced secretion. By introducing both Rac2 and Cdc42 simultaneously into permeabilised cells, I have obtained evidence suggesting that Cdc42 can interact with the Rac2 effector in addition to a second (possibly specific) effector.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AA</td>
<td>Arachidonic acid</td>
</tr>
<tr>
<td>ACK</td>
<td>Activated Cdc42-associated kinase</td>
</tr>
<tr>
<td>ARF</td>
<td>ADP-Ribosylation Factor</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine-5'-triphosphate</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CaM</td>
<td>Calmodulin</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine-3':5'- monophosphate</td>
</tr>
<tr>
<td>CAPS</td>
<td>Ca²⁺-dependent activator protein for secretion</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>cmc</td>
<td>Critical micelle concentration</td>
</tr>
<tr>
<td>cpm</td>
<td>Counts per minute</td>
</tr>
<tr>
<td>CRIB</td>
<td>Cdc42/Rac interactive binding domain</td>
</tr>
<tr>
<td>Csp</td>
<td>Cysteine string protein</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DFP</td>
<td>Diisopropyl phosphofluoridate</td>
</tr>
<tr>
<td>dpm</td>
<td>Disintegrations per minute</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediamine tetraacetic acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethyleneglycol</td>
</tr>
<tr>
<td></td>
<td>-bis(β-aminoethyl ether) N, N, N', N'-tetraacetic acid</td>
</tr>
<tr>
<td>FAK</td>
<td>Focal adhesion kinase</td>
</tr>
<tr>
<td>FcεRI</td>
<td>High affinity receptor for IgE</td>
</tr>
<tr>
<td>FOAD</td>
<td>Factor of Activation of Degranulation</td>
</tr>
<tr>
<td>FPLC</td>
<td>Fast protein liquid chromatography</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase activating protein</td>
</tr>
<tr>
<td>GDI</td>
<td>Guanine nucleotide dissociation inhibitor</td>
</tr>
<tr>
<td>GDP</td>
<td>Guanosine-5'-diphosphate</td>
</tr>
<tr>
<td>GEF</td>
<td>Guanine nucleotide exchange factor</td>
</tr>
<tr>
<td>GppNHp</td>
<td>Guanosine 5'-[β, γ-imido] triphosphate</td>
</tr>
<tr>
<td>GSH</td>
<td>Reduced glutathione</td>
</tr>
<tr>
<td>GST</td>
<td>Glutathione S-transferase</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine-5'-triphosphate</td>
</tr>
<tr>
<td>GTPγS</td>
<td>Guanosine-5'-O-(3-thiotriphosphate)</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>IgE</td>
<td>Immunoglobulin E</td>
</tr>
<tr>
<td>IP₃</td>
<td>Inositol 1, 4, 5-trisphosphate</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl β-D-thiogalactoside</td>
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MLCK  Myosin light chain kinase
mRNA  Messenger ribonucleic acid
MW  Molecular weight
NDPK  Nucleoside diphosphate kinase
NSF  N-ethyl maleimide sensitive fusion protein
PA  Phosphatidic acid
PAK  p21<sup>Cdc42/Rac1</sup>-activated kinase
PBS  Phosphate buffered saline
PC  Phosphatidylcholine
PC12 (cells)  Pheochromocytoma (cells)
pCa  -log<sub>10</sub> [Ca<sup>2+</sup>]
PI  Phosphatidylinositol
PI-3K  Phosphatidylinositol 3-kinase
PI-4K  Phosphatidylinositol 4-kinase
PIP  Phosphatidylinositol 4-phosphate
PIP<sub>2</sub>  Phosphatidylinositol 4, 5-bisphosphate
PIP<sub>3</sub>  Phosphatidylinositol 3, 4, 5-trisphosphate
PIP-5K  Phosphatidylinositol-4-phosphate 5-kinase
PI-TP  Phosphatidylinositol transfer protein
PKA  Protein kinase A
PKC  Protein kinase C
PLA<sub>2</sub>  Phospholipase A<sub>2</sub>
PLC  Phospholipase C
PLD  Phospholipase D
PMA  Phorbol myristate acetate
PMSF  Phenylmethylsulfonyl fluoride
PVDF  Polyvinylidene difluoride
RBL-2H3 (cells)  Rat basophilic leukaemia (cells)
SDS-PAGE  Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SNAP  Soluble NSF attachment protein
SNARE  SNAP receptor
TFA  Trifluoroacetic acid
TNF  Tumour necrosis factor
UDP  Uridine-5’-diphosphate
WASp  Wiskott-Aldrich syndrome protein
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CHAPTER 1

“INTRODUCTION”
INTRODUCTION

Mast Cells - General Physiology

Mast cells are secretory cells found on the mucosal and serosal surfaces of tissues throughout the body. They are involved in the immunological response known as immediate hypersensitivity or, more simply, the allergic response. Mast cells arise from precursors in the bone marrow, which are carried in the blood to the tissues where they mature under the influence of cytokines. When isolated for examination by transmission electron microscopy, they appear rounded (generally 6-13μm in diameter), and the cytoplasm is packed with about 1000 granules. The granules are the sites of storage of preformed inflammatory mediators, which are released by the mechanism of exocytosis into the extracellular environment when the cell is activated by the cross-linking of the high affinity receptors for IgE present on the cell surface (see later). These mediators include histamine, neutral proteases such as tryptase and chymase, acid hydrolases including β-hexosaminidase and β-galactosidase, and chemotactic factors such as TNFα (although it has been demonstrated that upon cell stimulation, not only is granular TNFα released, but additional TNFα is synthesised and subsequently released (Gordon and Galli, 1991). Also contained within the granules are proteoglycans, such as heparin and chondroitin, which comprise the structural component of the granule. They have glycosaminoglycan side chains with which the other mediators are thought to associate via ionic interactions to form protein-proteoglycan complexes which maintain the proteins in a stable configuration in the resting cell (Church and Caulfield, 1993).

Mast cells possess high affinity receptors for IgE in their plasma membranes, known as FcεR1. These receptors consist of four subunits, two α chains, a β, and a γ chain. The α subunits are the sites of IgE binding. The cells are activated immunologically by the crosslinking of IgE molecules by specific antigens (Segal et al., 1977), which brings the receptors into close proximity. There is also some evidence to suggest that the relative orientation of receptors upon cross-linking is an important factor in determining the magnitude of the stimulus delivered to the cell by IgE (Ortega et al., 1988). Receptor activation induces the fusion of the secretory granules with the plasma membrane allowing release of their contents to
the exterior. In addition, the stimulus results in the production of additional mediators from arachidonic acid liberated by the action of phospholipase A2 on membrane phospholipids. In this way, prostaglandins are produced via activation of the cyclo-oxygenase pathway, and leukotrienes via activation of the lipoxygenase pathway. Mast cells are also able to synthesise platelet activating factor (PAF) which is a potent chemoattractant for neutrophils and eosinophils, and is therefore important in the initiation of the inflammatory response. Other mast cell mediators have been postulated to play a similar role. It is the actions of both the pre-formed and the newly-formed mediators that give rise to the symptoms of the allergic reaction such as the bronchoconstriction which causes breathlessness in asthma. In some cases, these same mediators can be much more aggressive in their actions, causing the potentially fatal syndrome known as systemic anaphylaxis. Here, the mediators of the allergic response have widespread detrimental effects, targeting more than one organ, sometimes far from the site of the initial exposure to allergen. Systemic anaphylaxis is abrupt in onset, and its potentially lethal consequences include acute upper respiratory obstruction, bronchospasm, and shock with vascular collapse.

In sharp contrast to this destructive behaviour, mast cells also play a vital role in the amplification of acute inflammatory responses arising from non-immunological origins (Wershil et al., 1988). They have been shown to perform a crucial role in host defence against bacterial infection, since TNFα (tumour necrosis factor), released by mast cells in the vicinity of the infection, stimulates neutrophil influx to the affected area (Zhang et al., 1992). This appears to be vital for efficient bacterial clearance (Echtenacher et al., 1996; Malaviya et al., 1996). Hence, mast cells can be protective as well as destructive in their activities.

Although IgE crosslinking is the mechanism by which mast cells are activated by antigen, mast cells can also be activated via alternative routes. It is possible to activate mast cells using the complement peptides C3a and C5a, substance P, mastoparan, compound 48/80, and calcium ionophores (to name but a few). The ease with which mast cells can be isolated and handled, the ease with which one can measure the secreted materials (such as β-hexosaminidase), and the fact that
mast cell degranulation can be monitored under the microscope, has made them a useful model for studying the mechanisms underlying regulated exocytosis. The secreting mast cell line RBL-2H3 has also been used extensively in such studies (for review see (Beaven and Kassessinoff, 1997)).

Requirements for exocytosis

\( \text{Ca}^{2+} \)

It is widely accepted that \( \text{Ca}^{2+} \) regulates exocytosis from many types of secretory cell, and mast cells are no exception. Mast cells stimulated by the crosslinking of the receptors for IgE have been found to require extracellular \( \text{Ca}^{2+} \) in the range 0.1-1mM (Foreman and Mongar, 1972). The demonstration of a \( \text{Ca}^{2+} \) influx accompanying degranulation in mast cells stimulated with antigens (Foreman et al., 1973a), and the finding that secretion can be stimulated by application of \( \text{Ca}^{2+} \)-ionophores (Foreman et al., 1973b), provided an explanation for these findings, and subsequent work has firmly established that increases in intracellular \( \text{Ca}^{2+} \) occur alongside, and are indeed necessary for the activation of mast cells (and RBL-2H3 cells) (Foreman et al., 1977; Beaven et al., 1984b; Hide and Beaven, 1991). In contrast, it has been shown that cells stimulated by agents of the class which includes compound 48/80 have no requirement for \( \text{Ca}^{2+} \) in the stimulation of secretion (Douglas and Ueda, 1973). It is now recognised that \( \text{Ca}^{2+} \) increases are initiated and maintained via two distinct mechanisms. Upon mast cell stimulation, \( \text{Ca}^{2+} \) is released from intracellular inositol 1,4,5-trisphosphate (IP\(_3\))-sensitive stores, as evidenced by increased phosphatidylinositol breakdown (Cockcroft and Gomperts, 1979b; Beaven et al., 1984a; Cunha-Melo et al., 1987) and concomitant rises in IP\(_3\) which immediately precede the rise in cytoplasmic \( \text{Ca}^{2+} \) concentration (Pribluda and Metzger, 1987). Direct evidence has come from the demonstration that addition of IP\(_3\) does indeed cause release of \( \text{Ca}^{2+} \) from internal stores (Meyer et al., 1988; Matthews et al., 1989). The \( \text{Ca}^{2+} \) increase is maintained, upon depletion of the stores, by influx of external \( \text{Ca}^{2+} \) via a non-voltage activated \( \text{Ca}^{2+} \) channel (Matthews et al., 1989; Hoth and Penner, 1992; Kanner and Metzger, 1984; Kuno et al., 1989) which may be regulated by a GTP-binding protein (Matthews et al., 1989; Fasolato et al., 1993; Bird and Putney, 1993). In addition, in RBL-2H3 cells, it now appears that there is also a gated \( \text{Ca}^{2+} \)-permeable channel regulated by intracellular sphingolipids (Kindman
et al., 1994; Mao et al., 1996) and its existence is supported by independent findings that sphingosine kinase stimulates intracellular Ca\(^{2+}\) mobilisation via sphingosine-1-phosphate production (Choi et al., 1996).

Cell permeabilisation techniques which allow direct manipulation of the composition of the cytosol have been widely used in the investigation of exocytotic mechanisms. Initially, by permeabilising mast cells by application of extracellular ATP (strictly, ATP\(^{4+}\)), it was found that Ca\(^{2+}\), buffered at concentrations in the micromolar range, is necessary to stimulate secretion (Bennett et al., 1981). Subsequent studies using alternative permeabilising agents, such as Sendai virus (Gomperts et al., 1983) and then, streptolysin-O (Howell and Gomperts, 1987) have confirmed and extended these initial findings. More recently, it has become clear that the key step of regulation in these cells is the activation of a GTP-binding protein since provision of a guanine nucleotide is not only necessary but by itself can be sufficient for secretion. Ca\(^{2+}\) is better regarded as a modulator but this still implies the involvement of a Ca\(^{2+}\)-binding protein (C\(_E\)) which has yet to be identified (Lillie and Gomperts, 1992b; Lillie and Gomperts, 1992a).

In many other secretory cell types Ca\(^{2+}\) is not only absolutely necessary, alone it is sufficient to trigger exocytosis. However, different cell types and even different vesicle types (e.g. in neuronal synapses) exhibit differing Ca\(^{2+}\) requirements to stimulate secretion. These differences must surely reflect the involvement of different Ca\(^{2+}\)-binding proteins (or different isoforms of Ca\(^{2+}\)-binding proteins) in the regulatory pathways to secretion in each cell type. For example, synaptic vesicle exocytosis appears to require a “low affinity” Ca\(^{2+}\) sensor (since 190\(\mu\)M Ca\(^{2+}\) is required for half maximal stimulation of exocytosis, as measured using a flash photolysis technique (Heidelberger et al., 1994)), whilst secretion from adrenal chromaffin cells requires a “high affinity” Ca\(^{2+}\) sensor (since only 10-20\(\mu\)M Ca\(^{2+}\) is sufficient to achieve a half maximal initial rate of exocytosis, again measured using flash photolysis (Heinemann et al., 1994), as reviewed in (Burgoyne and Morgan, 1995; Morgan and Burgoyne, 1997). Ca\(^{2+}\)-binding proteins postulated to act as Ca\(^{2+}\) sensors in the regulation of secretion are discussed later (see “Proteins involved in the regulation of exocytosis”).
Guanine Nucleotides

Secretion from intact mast cells requires the presence of GTP regardless of the nature of the stimulus. Following depletion of GTP in intact cells by treatment with mycophenolic acid or ribavirin (inhibitors of IMP dehydrogenase (Yamada et al., 1988; Sweeney et al., 1972)), mast cells fail to respond to stimulation by ligands which aggregate the receptors for IgE, or to application of receptor-mimetic agents such as mastoparan or compound 48/80, or even to Ca\(^{2+}\)-carrying ionophores (Mulkins et al., 1992; Wilson et al., 1989; Marquardt et al., 1987). This information, taken together with the finding that it is possible to induce secretion from permeabilised cells by provision of the non-hydrolysable analogue of GTP, guanosine 5'-O-(3-thiotriphosphate) (GTP\(_\gamma\)S) in the effective absence of Ca\(^{2+}\) (Howell et al., 1987; Lillie and Gomperts, 1992b; Larbi and Gomperts, 1996) is evidence for an essential role for a GTP-binding protein (G\(_E\)) at a late stage in the signalling pathway regulating the exocytotic event. However, it is apparent that, depending on the nature of the stimulus, different classes of GTP-binding proteins are involved. Cells pretreated with Pertussis toxin fail to respond to mastoparan or compound 48/80, but remain fully sensitive to stimulation by specific antigens or Ca\(^{2+}\)-ionophores (Saito et al., 1987).

The idea of a G\(_E\) originally arose from experimental results obtained with neutrophils, which when permeabilised were also shown to secrete in response to GTP\(_\gamma\)S in the effective absence of Ca\(^{2+}\) (<10\(^{-10}\)M). The guanine nucleotide was proposed to act at two sites, the first being the activation of polyphosphoinositide phosphodiesterase (PPI-pde, the enzyme now commonly referred to as phospholipase C), the second being at a late stage in the exocytotic process, which was called G\(_E\) (Barrowman et al., 1986). The related HL60 cells, when permeabilised, exhibit enhanced secretion in response to application of guanine nucleotides alongside Ca\(^{2+}\) (Stutchfield and Cockcroft, 1988), as do pancreatic acinar cells (Padfield et al., 1991) and platelets (Haslam and Davidson, 1984; Knight and Scrutton, 1987) (the only difference being that in platelets, of the guanine nucleotides tested, GTP\(_\gamma\)S was found in one study to induce Ca\(^{2+}\)-independent secretion in addition to enhancing Ca\(^{2+}\)-dependent secretion (Haslam and Davidson, 1984)). In contrast, permeabilised parathyroid cells exhibit
Ca\textsuperscript{2+}-independent secretion in response to both GTP\textgamma{}S and GppNHp (Oetting et al., 1986; Matovcik et al., 1997). There is controversy surrounding the role of GTP-binding proteins in the regulation of secretion from adrenal chromaffin cells. Two studies have shown a stimulation of Ca\textsuperscript{2+}-independent secretion by GTP\textgamma{}S and GppNHp (Bittner et al., 1986; Morgan and Burgoyne, 1990b) whilst others find that GTP\textgamma{}S exerts little effect on Ca\textsuperscript{2+}-independent secretion, but can enhance Ca\textsuperscript{2+}-dependent secretion (Burgoyne et al., 1989; Bader et al., 1989). This latter finding was again at odds with an earlier report suggesting that whilst GTP analogues can indeed enhance the extent of Ca\textsuperscript{2+}-dependent secretion, this is apparent only at sub-optimal Ca\textsuperscript{2+} concentrations (Morgan and Burgoyne, 1990b). Yet another study suggested that Ca\textsuperscript{2+}-dependent secretion can be inhibited by preincubation of cells with GTP\textgamma{}S (Knight and Baker, 1985), and although a similar inhibition was also found by others, the inhibitory nucleotide was found to be GTP, not GTP\textgamma{}S (Morgan and Burgoyne, 1990b). Hence, whilst GTP-binding proteins appear to fulfil modulatory roles in chromaffin cell exocytosis, their mechanism of action remains far from certain.

Overall it is clear that GTP-binding proteins have some kind of role in exocytotic regulation in many secretory cells and this probably differs according to the cell type. Of course, most of the initial reports gave little clue concerning the classes of GTP-binding protein(s) involved in secretory regulation, but subsequent work has explored the possible role(s) of specific proteins in this process, and this is described in detail later (see “GTP-binding proteins in the regulation of exocytosis”).

**ATP**
Treatment of mast cells with metabolic inhibitors prevents secretion induced by all forms of external stimulus. It is generally accepted that this is due to the depletion of intracellular ATP. However, secretion from permeabilised mast cells stimulated by the dual effectors Ca\textsuperscript{2+} plus GTP\textgamma{}S (Howell et al., 1987; Koopmann and Jackson, 1990), or by intracellular microinjection of GTP\textgamma{}S (Tatham and Gomperts, 1991) occurs in the absence of ATP. It follows that a phosphorylation reaction does not constitute an essential event in the signalling pathway leading to granule release. Under conditions where mast cells are permeabilised and
stimulated in a Na-glutamate-based buffer (instead of an NaCl-based buffer),
exocytosis can be stimulated by GTPγS alone. On the other hand, when Ca^{2+} is
used as the stimulus it is necessary to supply ATP and this can be enhanced by
low concentrations (and then inhibited by higher concentrations) of GDP. This
suggests that the role of ATP under these conditions is in the maintenance of GTP
by transphosphorylation catalysed by nucleoside diphosphate kinase (Lillie and
Gomperts, 1992b). Secretion elicited in this manner is also inhibited by inhibitors
of protein kinase C (PKC) (Churcher and Gomperts, 1990), implying that the need
for ATP reflects a role for PKC-mediated phosphorylations in the stimulation of
secretion by Ca^{2+} alone.

Although ATP is not essential for secretion from mast cells when stimulated by
the dual effector system (Ca^{2+} plus GTPγS), it has been found to exert profound
modulatory effects. For example, the effective affinity (EC_{50}) of each of the two
effectors is enhanced in the presence of ATP (Howell et al., 1987; Koopmann and
Jackson, 1990; Cockcroft et al., 1987; Lillie et al., 1991). The initial suggestion
that this enhancement is mediated by protein kinase C (PKC) (Cockcroft et al.,
1987), is well supported by the findings that phorbol myristate acetate (PMA - an
activator of PKC) potentiates the enhancement of effector affinity (Koopmann and
Jackson, 1990), whilst AMG-C_{16}, a glycerol diether analogue of the PKC activator
diacylglycerol (a diester) (Kramer et al., 1989), is inhibitory (Lillie et al., 1991).
Hence it appears that whilst PKC plays no direct role in the stimulation of
secretion from permeabilised mast cells, it certainly exerts modulatory effects and
these become compulsory when secretion is stimulated by Ca^{2+} alone (Churcher
and Gomperts, 1990). The role of PKC in mast cell secretion is discussed in more
detail later (see “Proteins involved in the regulation of exocytosis”).

Another effect of ATP is to prolong the period during which mast cells remain
responsive to stimulation (by the dual stimulus) following permeabilisation. As
will be described in detail later (see “The rundown assay”), mast cells become
refractory to stimulation (by Ca^{2+} and GTPγS) as the time between
permeabilisation and application of the stimulus is extended. This occurs rapidly
(within 5min) when the cells are permeabilised in the absence of ATP (Howell et
al., 1989). Activity can, however, be restored by the late addition of ATP,
indicating that the maintenance of a phosphorylated state is necessary for secretion to occur. Again, PKC has been implicated in this process (Lillie et al., 1991; Howell et al., 1989). The loss of responsiveness was found to be due to a decline in the "effective affinity" for Ca\(^{2+}\), and, after the readdition of ATP, secretion can be elicited by Ca\(^{2+}\) alone. However, in cells pretreated with okadaic acid, an inhibitor of protein phosphatases, or PMA, guanine nucleotide remains an absolute requirement. From these data, it was suggested that activation of exocytosis might involve a dephosphorylation step regulated by a GTP-binding protein (Churcher et al., 1990b). An independent demonstration of the inhibition of mast cell secretion by okadaic acid (Peachell and Munday, 1993) lent further credence to this idea, and it was originally supported by the finding that cells permeabilised and stimulated in the presence of ATP only commence secretion after a delay, which can be eliminated by withholding ATP or substituting the stable analogue AppNHp (Tatham and Gomperts, 1989). The idea was that ATP caused a phosphorylated state to be maintained, but this was questioned in later work when it was shown that the ATP-dependent onset delays are also manifest when Mg\(^{2+}\) (essential for all known kinase reactions) is absent from the system (Lillie et al., 1991). So the role of a possible dephosphorylation step in the signalling pathway leading to mast cell exocytosis remains unproved. However, there is some evidence for a dephosphorylation step in the regulation of exocytosis from other cell types, such as *Paramecium tetraurelia* (Momayezi et al., 1987) and pancreatic acini (Jena et al., 1991).

An important discovery regarding the role of ATP came with the finding that regulated secretion from digitonin-permeabilised adrenal chromaffin cells and from semi-intact PC12 cells can be resolved into two stages with distinct effector requirements. The "priming" stage has been found to be Mg,ATP-dependent, whilst the "triggering" stage (which is preceded by "priming"), is Ca\(^{2+}\)-dependent but independent of Mg,ATP (Holz et al., 1989; Hay and Martin, 1992; Bittner and Holz, 1992). Each stage is reliant on a distinct set of cytosolic proteins. Three proteins regulating ATP-dependent priming in semi-intact PC12 cells have been described (Hay and Martin, 1992). Two of these have been isolated and identified as phosphatidyl-inositol transfer protein (PITP) (Hay and Martin, 1993), and type I phosphatidylinositol-4-phosphate 5-kinase (PIP-5K) (Hay et al., 1995). Their activities have been demonstrated to synergise in a priming assay, consistent with
their sequential activation (Hay and Martin, 1993; Hay et al., 1995), and their
identification as exocytic regulators confirmed earlier reports where inositol
phospholipids were postulated to be important in the regulation of exocytosis
(Eberhard et al., 1990). Hence it appears that in chromaffin cells and PC12 cells,
an important role of ATP is in the phosphorylations catalysed by inositol lipid
kinases to maintain polyphosphoinositide levels. It seems likely that priming and
triggering represent universal stages in exocytosis from all cells exhibiting a
regulated exocytotic pathway, but such events have not yet been directly
demonstrated in mast cells.

cAMP
In general, secretion from mast cells is associated with falls in intracellular cAMP
levels. Elevation of the intracellular concentration of cAMP is understood to be
inhibitory to exocytosis (Sullivan et al., 1975; Shores and Mongar, 1980) and
medically, drugs such as theophylline (inhibitor of cAMP phosphodiesterase) and
salbutamol (β-adrenergic agonist) are widely used to suppress the release of
allergic mediators such as histamine. However, transient elevations of cAMP have
been recorded in the seconds following stimulation with IgE directed ligands
(Carpenter and Cantley, 1996; Parsons, 1996) and the role of cAMP in the
regulation of mast cell exocytosis has not been satisfactorily explained (reviewed
in (Alm and Bloom, 1982)). It appears to be modulatory, but its role is not that of
a simple activator or inhibitor, and therefore remains obscure. In contrast, cAMP
has been found to be a stimulator of exocytosis in adrenal chromaffin cells
(Morgan et al., 1993), pancreatic acini (O'Sullivan and Jamieson, 1992), and
parotid acini (Takuma and Ichida, 1991).

Proteins involved in the regulation of mast cell exocytosis

The “rundown” assay
It is appropriate here to introduce the concept of “rundown”, already mentioned in
passing (see under “ATP”). This has been exploited in the quest to identify protein
regulators of secretion, and is similarly exploited in much of the work presented in
this thesis (see Chapter 2). Permeabilisation in the presence of normally
impermeant low molecular weight solutes (such as Ca^{2+} and guanine nucleotides)
allows these to be tested as acute stimulators or modulators of secretion. However, a possible consequence of permeabilisation, depending on the method used, is the leakage of intracellular proteins. For mast cells permeabilised by streptolysin-O, the resulting secretory response is much diminished if the stimulus is initially withheld and then applied some minutes after permeabilisation (Howell and Gomperts, 1987; Howell et al., 1989). This decline in responsiveness, now called "run-down" has been ascribed to the leakage of intracellular proteins through the membrane lesions created by the permeabilising agent. (NB. An experiment illustrating the progressive decline in responsiveness with time is illustrated in Chapter 2, Figure 2.1). Certainly, soluble proteins do leak through the lesions created by reagents such as streptolysin-O and digitonin (Howell and Gomperts, 1987; Gomperts et al., 1987b; Koffer and Gomperts, 1989; Sarafian et al., 1987). Depending on the permeabilising agent and conditions, run-down occurs at different rates and this can be related to the dimensions of the permeability lesions generated and the rate of protein leakage. For example, the lesions generated by ATP<sup>+</sup> (Cockcroft and Gomperts, 1979a) are much smaller than those generated by streptolysin-O. The leakage of soluble actin from ATP<sup>+</sup> permeabilised mast cells persists over 60 minutes. By contrast, the leakage of actin induced by streptolysin-O is effectively complete after 15 minutes (Koffer and Gomperts, 1989) and the onset and subsequent rate of loss of responsiveness is much more rapid. Also, it has been shown that the smaller the protein, the more promptly it leaks, as might be expected. For example, phosphoglycerate kinase (MW 45 kDa) leaks more rapidly from streptolysin-O permeabilised cells than lactate dehydrogenase (MW 140kDa) (Gomperts et al., 1987b). From this, it follows that it should be possible to introduce exogenous proteins into the cytosol over a similar period of time.

The loss of sensitivity to stimulation following permeabilisation of secretory cells and its recovery by incubation of these "run-down" cells with specific proteins has been used by a number of groups as the basis of a procedure to enable identification of proteins which may be exocytotic regulators. The central assumption is that proteins which enhance or inhibit stimulated secretion in cells which have been allowed to run down by permeabilising initially in the absence of a stimulus, are likely to at least similar, if not identical to the actual regulators operating in vivo.
Proteins identified as putative exocytotic regulators in this manner using various cell types include p145/CAPS (Walent et al., 1992; Nishizaki et al., 1992), 14-3-3 protein (Exo1) (Morgan and Burgoyne, 1992a; Wu et al., 1992), the catalytic subunit of PKA (Exo2) (Morgan et al., 1993), annexin II (Ali et al., 1989; Ali and Burgoyne, 1990; Sarafian et al., 1991), and SNAPS (Morgan and Burgoyne, 1995a; Sudlow et al., 1996). The possible roles of these, and other proteins in exocytotic regulation is discussed in this section.

**Tyrosine kinases**
Several proteins have been found to undergo phosphorylation on tyrosine residues following stimulation of the RBL-2H3 FceR1 (Benhamou et al., 1990; Kawakami et al., 1992; Lin et al., 1994), (including phospholipases C\(\gamma\)1 (Park et al., 1991; Yamada et al., 1992) and \(\gamma\)2 (Beaven and Kassessinoff, 1997)), and this occurs before any elevation in the intracellular concentration of Ca\(^{2+}\) becomes detectable (Benhamou et al., 1990; Kawakami et al., 1992). These phosphorylations, now known to be mediated by non-receptor tyrosine kinases such as the gene products of Lyn and Syk, appear to be necessary for receptor stimulated secretion in mast cells and RBL-2H3 cells (Kawakami et al., 1992; Lin et al., 1994; Yamada et al., 1992), and it has been proposed that crosslinking of the receptors for IgE leads to the sequential activation of Lyn and then Syk, which in turn phosphorylate and activate PLC\(\gamma\)1 (Jouvin et al., 1994).

**Protein kinase C (PKC)**
In RBL-2H3 cells, inhibition of PKC has been demonstrated to cause inhibition (sometimes only partial) of secretion (Benhamou et al., 1990; Yamada et al., 1992), and in other studies PKC activation is insufficient to stimulate RBL-2H3 secretion (Lin and Gilfillan, 1992; Ali et al., 1996). In another study, PKC inhibition had no apparent effect on secretion (Cunha-Melo et al., 1989). These data indicate that PKC plays a modulatory, non-essential role in these cells.

One action of PKC following receptor-mediated RBL-2H3 stimulation is the phosphorylation of myosin light and heavy chains, the time course of which parallels the time course of histamine secretion (Ludowyke et al., 1989). It was
later found that there is a correlation between this PKC-mediated phosphorylation and another MLCK-mediated phosphorylation (which also occurs upon cell stimulation) and the extent of secretion, leading to the suggestion that both phosphorylations are required for receptor-stimulated secretion from these cells (Choi et al., 1994).

Although it is not known which isozymes of PKC are responsible for these particular phosphorylations, PKCs β and δ were found to reconstitute secretion in run down, washed RBL-2H3 cells. These effects were apparent only in the presence of Ca\(^{2+}\), implicating these isozymes in the regulation of Ca\(^{2+}\)-dependent secretion from these cells (Ozawa et al., 1993a). There is also some indirect evidence for an additional role for PKCδ in the down-regulation of Fc\(_{ɛ}\)R1 receptor activation by receptor endocytosis (Germano et al., 1994), and PKCs α and ε were later found to mediate the feedback inhibition of phospholipase C\(_{γ1}\) in RBL-2H3 cells (Ozawa et al., 1993b).

**Phospholipases**

*Phospholipase C*

Fc\(_{ɛ}\)R1 receptor cross-linking results in the tyrosine phosphorylation (and activation) of phospholipase C\(_{γ1}\) (PLC\(_{γ1}\)) (Park et al., 1991) and \(γ2\) (Beaven and Kassessinoff, 1997) resulting in the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP\(_2\)) (Park et al., 1991) in RBL-2H3 cells. It is already well documented that hydrolysis of PIP\(_2\) leads to the generation of IP\(_3\) and diacylglycerol (DAG), the former being crucial for stimulating the release of Ca\(^{2+}\) from intracellular stores (Meyer et al., 1988; Matthews et al., 1989), the latter being necessary for PKC activation (Hug and Sarre, 1993). As both these events are involved in the regulation of secretion from mast cells, then it appears that PLC\(_γ\) is almost certainly a regulator in this process.

*Phospholipase D*

Phospholipase D (PLD) is activated upon Fc\(_{ɛ}\)R1 receptor stimulation in RBL-2H3 cells (Lin et al., 1991), and this is dependent upon the concomitant increase in intracellular Ca\(^{2+}\) concentration and PKC activity (Lin and Gilfillan, 1992). PLD
was found to predominate over PC-specific PLC in the hydrolysis of phosphatidylcholine (PC) in activated mast cells (Dinh and Kennerly, 1991). However, the increase in PLD activity and the associated increase in PKC activity are insufficient to cause secretion in themselves (Ali et al., 1996; Beaven and Kassessinoff, 1997), and there still exists no direct evidence supporting a role for PLD in the regulation of exocytosis by these cells. In differentiated HL60 cells, though, a correlation between PLD activity and secretory activity has been reported, and it was concluded that the product resulting from PLD activation, phosphatidic acid, acts as a second messenger in the stimulation of the secretory pathway but is not required for exocytosis per se (Stutchfield and Cockcroft, 1993). There is also some evidence for an involvement of PLD in exocytosis from pancreatic islets (Metz and Dunlop, 1990), and platelets (Coorssen and Haslam, 1993), although in the case of platelets, this appears to be modulatory rather than essential (Coorssen, 1996).

**Phospholipase A\(_2\)**

Stimulation of both intact and permeabilised mast cells or RBL-2H3 cells leads to activation of phospholipase A\(_2\) (PLA\(_2\)) with the generation of arachidonic acid (AA) (Hirasawa et al., 1995a; Churcher et al., 1990a). The early suggestion that this enzyme plays a direct role in secretory regulation (Chi et al., 1982; Nagai et al., 1991) was questioned when it was found that the two activities can be separated (Churcher et al., 1990a; Collado-Escobar et al., 1989). Subsequent studies have revealed that activation of Syk, a consequence of IgE-R crosslinking, leads to the phosphorylation of Vav (a GDP/GTP exchange factor for the small GTP binding proteins Rac (Crespo et al., 1996; Crespo et al., 1997; Teramoto et al., 1997) and possibly Ras (Gulbins et al., 1993; Gulbins et al., 1994a; Gulbins et al., 1994b), although this latter activity is disputed (Bustelo et al., 1994; Khorasavi-Far et al., 1994; Han et al., 1997)) and activation of mitogen-activated protein kinase (MAPK). This latter event results in activation of PLA\(_2\), leading to AA release (Hirasawa et al., 1995b; Hirasawa et al., 1995a). This pathway to AA release is independent of PKC and is distinct from the pathway controlling exocytosis in these cells (Hirasawa et al., 1995a), thus confirming earlier indirect indications that PLA\(_2\) activity is dispensable in the activation of mast and RBL-2H3 cell secretion. There is evidence, however, that PLA\(_2\) may be a positive
regulator of exocytosis in some other cell types, since PLA$_2$ inhibition has been demonstrated to inhibit secretion from pancreatic islets (Loweth et al., 1996), and arachidonic acid appears to stimulate secretion from anterior pituitary cells (Chang et al., 1986), and GH$_3$ cells (Kolesnick et al., 1984). Exocytosis from adrenal chromaffin cells appears to have no requirement for PLA$_2$, as secretion induced by Ca$^{2+}$ and also GTP analogues occurs regardless of arachidonic acid levels (Morgan and Burgoyne, 1990b; Morgan and Burgoyne, 1990a).

**Phosphatidylinositol 3-kinase (PI 3-kinase)**
Although this enzyme appears to be a component of many signalling systems (reviewed in (Kapeller and Cantley, 1994), there are only limited data available regarding a role in the regulation of mast cell secretion. Certainly, wortmannin, an inhibitor of PI 3-kinase, inhibits IgE-mediated secretion from both mast cells and RBL-2H3 cells (Yano et al., 1993; Marquardt et al., 1996), though there must be some doubt regarding the conclusion as wortmannin can inhibit secretion independently of its PI 3-kinase activity (Gao et al., 1996; Oda et al., 1997).

It has been proposed that PI 3-kinase acts early in the signalling pathway(s) activated as a result of receptor cross-linking in mast cells, and has both protein and lipid kinase activities. It is suggested that it may phosphorylate multiple substrates, making it a point of divergence of many signalling pathways leading to distinct cellular responses, including secretion (Barker et al., 1995). PI 3-kinase has been postulated to be a secretory regulator in MIN6 (a pancreatic β cell line) cells (Hagiwara et al., 1995), but once again, this conclusion is based on studies using the less-than-specific wortmannin as an inhibitor and so should probably be treated with some caution.

**SNAPs, SNAREs and associates**
Although much of the work on these proteins has been concerned with the constitutive secretory pathway, the current state of play is summarised here because it is becoming increasingly clear that many of the mechanisms may well be similar if not identical to those underlying fusion of secretory vesicles with the plasma membrane in regulated exocytosis.
The first components of the so-called vesicle fusion complex to be identified were NSF (N-ethyl maleimide (NEM) sensitive fusion protein (Malhotra et al., 1988; Orci et al., 1989; Block et al., 1988), and SNAPs (soluble NSF attachment proteins), through which NSF interacts with binding sites (SNAP receptors, or SNAREs) on the corresponding membranes. (Weidman et al., 1989; Whiteheart et al., 1992). NSF has ATPase activity (Whiteheart et al., 1994) which is enhanced by SNAP (Morgan et al., 1994). NSF-catalysed ATP hydrolysis has been results in the dissociation of the NSF/SNAP/SNARE complex, and this is blocked by ATPγS (Wilson et al., 1992). This has in turn been exploited in the affinity purification of SNARE proteins from brain tissue. Three proteins have been identified: syntaxin and SNAP-25 (synaptosome associated protein of MW 25kDa, not to be confused with the SNAPs already mentioned), localised to presynaptic membranes, and VAMP (vesicle associated membrane protein, also known as synaptobrevin), localised to synaptic vesicle membranes. The finding that different SNARES are situated on donor and acceptor membranes led to the SNARE hypothesis which states that each transport vesicle contains a specific protein (v-SNARE) on its surface which determines which membrane it will ultimately bind and fuse. For every v-SNARE there exists a corresponding t-SNARE on the target membrane, so conferring specificity to ensure the correct targeting of each vesicle (Sollner et al., 1993b). It has recently emerged, however, that the t-SNAREs syntaxin and SNAP-25 are also present on vesicular membranes (Walch-Solimena et al., 1995), which throws some doubt on the idea of SNAREs specific to vesicle or target membranes.

Proteins which “block” either t- or v-SNAREs have been identified as regulators of membrane fusion. n-Sec1 (also known as munc 18 or rbSec1) binds to the t-SNARE syntaxin, but does not form part of the SNARE complex and inhibits VAMP and SNAP-25 binding to syntaxin (Garcia et al., 1994; Garcia et al., 1995; Pevsner et al., 1994b; Pevsner et al., 1994a). Synaptophysin appears to be a regulatory protein for VAMP as it destabilises VAMP/t-SNARE interactions (Edelmann et al., 1995), and is found associated with VAMP on synaptic vesicle membranes (Calakos and Scheller, 1994).

In addition to the proteins directly associated with NSF, GTPases of the Rab series also appear to be required for fusion, again probably in the determination of
donor-acceptor specificity (reviewed in (Pfeffer, 1994)). Although their precise
mechanism of action is still unknown, several studies have yielded evidence that
these proteins may facilitate SNARE complex formation by promoting
deprotection of the t-SNARE (Dascher et al., 1991; Brennwald et al., 1994;
Sogaard et al., 1994). Another fairly new idea is that so-called “Velcro” proteins
aid fusion by linking v- and t-SNAREs thus bringing them together. One possible
candidate is Rabaptin 5 which binds directly to Rab5 on endosomes to facilitate
endosome-endosome fusion (Stenmark et al., 1995).

It should be emphasised that since the early demonstrations of a role for NSF in
docking and fusion, some doubts have arisen as to whether it really is a fusion
protein. It was found that removal of α–SNAP (usually active at the same time as
NSF) does not inhibit subsequent fusion, and that NSF then remains
membrane-bound, indicating the existence of another NSF binding site besides
α–SNAP. In addition, donor and acceptor membranes which have proceeded past
the NSF/α-SNAP dependent stage are still able to undergo fusion, implying that
NSF is required only for very early events in the fusion process (Mayer et al.,
1996). It has been postulated that NSF may serve primarily as a mediator of
rearrangements in SNARE interactions, not as a fusogen (Morgan and Burgoyne,
1995b), and this idea has been further supported by the demonstration that NSF
and SNAP can act on single SNAREs, and are not required for SNARE complex
assembly or fusion. This indicates that they may activate, or “prime” SNAREs
before complex assembly and fusion (Nichols et al., 1997).

By far the strongest evidence supporting a role for SNAPs and SNAREs in
regulated exocytosis has been the demonstration that the tetanus and botulinum
toxins, which inhibit exocytosis in some cell types (e.g. (Bittner and Holz, 1988;
Bittner et al., 1989; Ahnert-Hilger and Weller, 1993; Gaisano et al., 1994; Penner
et al., 1986; Link et al., 1992)), exert their effects by cleaving specific SNARE
proteins. The tetanus and botulinum toxins B, D, F and G cleave
VAMP/synaptobrevin at single sites, whilst botulinum toxins A and E cleave
SNAP-25 at two distinct sites. Botulinum toxin C cleaves syntaxin, but the precise
site of cleavage remains unidentified (Schiavo et al., 1992; Montecucco and
Schiavo, 1994). The demonstration of the presence of substrates for the tetanus
and botulinum neurotoxins in cell types susceptible to blockage of secretion provides additional evidence supporting an essential role for the proteins in regulated exocytosis (Sollner et al., 1993a; Sollner et al., 1993b; Hodel et al., 1994; Roth and Burgoyn, 1994). However, it seems that SNAREs may not be a universal requirement for regulated exocytosis, as tetanus and botulinum A toxins are without effect on amylase secretion from permeabilised exocrine pancreatic acinar cells (Stecher et al., 1992). However, this may simply reflect the presence of SNARE isoforms which lack the specific sites cleaved by the toxins.

Three isoforms of SNAP have been identified, α, β, and γ SNAPs (Clary and Rothman, 1990), and it has been suggested that α and β isoforms fulfil unique roles in the control of regulated exocytosis (Schiavo et al., 1995). However, this hypothesis has been questioned by the finding that all three isoforms are capable of stimulating exocytosis from permeabilised, run down adrenal chromaffin cells (although γ was effective in only 50% of tests in this study) (Morgan and Burgoyn, 1995a) with α and β exerting indistinguishable effects when directly compared at a later date (Sudlow et al., 1996). Despite these contradictions, these data (and others, e.g. (DeBello et al., 1995)) do imply a role for SNAPs as exocytotic regulators, and additional confirmation has come with the demonstration that NSF (plus α–SNAP) appears to act during the Mg.ATP-dependent priming step in regulated exocytosis previously discussed (see under “ATP”) (Banerjee et al., 1996a). This confirmed earlier data showing that α–SNAP was active only in priming of chromaffin cells, and was inactive during triggering (Chamberlain et al., 1995), and this is also consistent with the hypothesis (mentioned earlier) that NSF and SNAP act as priming agents rather than as fusogents in the processes of membrane-membrane fusion.

Other proteins have also been implicated in the regulation of membrane fusion. SNAP-25 has recently been shown to act at a late Ca^{2+}-dependent step in PC12 cell exocytosis, beyond the stage of priming and vesicle docking (Banerjee et al., 1996b). Synaptophysin (the VAMP “blocking” protein) also appears to be involved in the control mechanisms underlying regulated exocytotic fusion (Alder et al., 1992a; Alder et al., 1992b), although it may not be essential (Eshkind and Leube, 1995). The localisation of the syntaxin “blocking” protein n-Sec1 to
chromaffin cell granules is consistent with a role for this protein in regulated secretion (Hodel et al., 1994). Rab proteins have also been implicated, and this is discussed in detail later (see “GTP-binding proteins in the regulation of exocytosis - Rab3”). In truth, new data regarding possible roles for individual proteins in regulated exocytosis are constantly emerging and previously held ideas are inevitably being questioned (see (Bock and Scheller, 1997)).

One potentially very interesting finding has been the discovery of a complex of proteins, termed the “exocyst”, which appears to be required for secretion in the budding yeast *Saccharomyces cerevisiae* (TerBush et al., 1996). This comprises seven proteins, and is localised to the tip of the bud, which is the predominant site of exocytosis. None of the proteins shows significant homology to any well characterised protein, and in addition, none of them appear to be required for any other steps of the secretory pathway. A similar complex has been isolated from brain tissue, and here the complex consists of eight proteins. Again, it is localised to the plasma membrane (in nerve terminals), and it co-immunoprecipitates with syntaxin (Hsu et al., 1996). Although the significance of these findings has yet to be fully elucidated, it may be that a unique fusion complex is involved in regulated exocytosis.

**Synaptotagmin**

Synaptotagmins are abundant synaptic vesicle proteins which contain two C2 domains homologous to those in present in PKC and which are postulated to mediate binding to Ca\(^{2+}\) and phospholipids (though not all of the eight isoforms identified actually bind either component) (Li et al., 1995). Initial indications that synaptotagmin associates with syntaxin (Bennett et al., 1992), calcium channels (Leveque et al., 1992) and with the α-latrotoxin receptor (Petrenko et al., 1991), and the finding that anti-synaptotagmin antibodies or peptides cause inhibition of secretion from PC12 cells and squid giant synapses (Elferink et al., 1993; Bommert et al., 1993) make this protein (the Ca\(^{2+}\)-binding isoforms) the favoured candidate for the Ca\(^{2+}\) sensor in synaptic vesicle and endocrine cell exocytosis. However, it appears that synaptotagmin may participate not only in exocytotic fusion, but also in endocytotic fusion (Li et al., 1995; Jorgensen et al., 1995).
It was suggested that synaptotagmin acts as a "clamp", acting to prevent membrane fusion in the absence of a triggering signal by binding to the SNARE complex and preventing the access of SNAP to initiate fusion (Sollner et al., 1993b; Sollner et al., 1993a). Subsequent studies now indicate that synaptotagmin acts not as a physical barrier to fusion but as a Ca^{2+} sensor (Geppert et al., 1994b; Broadie et al., 1994; Littleton et al., 1994). In PC12 cells it appears that synaptotagmin is not essential for secretory regulation, although this does not exclude the possibility that it plays some modulatory role (Shoji-Kasai et al., 1992), whilst other studies have shown that the absence of synaptotagmin impairs but does not abolish synaptic transmission, leading to the idea that another Ca^{2+} sensor operates and functions in a similar role (possibly alongside synaptotagmin)(Nonet et al., 1993; Broadie et al., 1994; Popov and Poo, 1993). Taken together, it appears that synaptotagmin has an important, but non-essential modulatory role, at least in synaptic vesicle and endocrine cell exocytosis. So far we have no data concerning a possible role for this protein in the exocytotic mechanisms of mast cells (and other related cells of haematopoietic origin).

**p145/CAPS (Ca^{2+}-dependent activator protein for secretion)**

Purified on the basis of its ability to reconstitute secretion from mechanically permeabilised, run down PC12 cells, the restricted expression of this protein in those tissues having a regulated secretory pathway suggests a specific role for CAPS as a regulator of exocytosis. CAPS is a Ca^{2+}-dependent phospholipid binding protein, and its activity as a promoter of exocytosis is potentiated when phosphorylated by PKC (Walent et al., 1992; Nishizaki et al., 1992). CAPS appears to act during the Ca^{2+}-dependent "triggering" step in regulated exocytosis from ATP-primed PC12 cells (Martin et al., 1995) and for this reason it must be considered a candidate for the "high affinity" Ca^{2+} sensor mediating exocytosis in these and adrenal chromaffin cells. It is a cytoskeletal protein, binding to F-actin and tropomyosin in a Ca^{2+}-dependent manner, with an EC_{50} reflecting the requirement for the stimulation of secretion in semi-intact PC12 cells. This has prompted the suggestion that CAPS may exert its effects by removing the cytoskeletal "clamp" that (possibly) exists as a barrier to fusion (Martin et al., 1995) (see "Cytoskeleton and exocytosis"). However, the finding that mast cell
exocytosis can proceed unimpeded when all cytoskeletal changes have been blocked (see later) must throw some doubt upon this as a universal mechanism of exocytotic regulation, although in the future CAPS may be shown to be involved in mast cell regulation as well. At present, the available data certainly support a role for this protein as a Ca^{2+} receptor in PC12 cells, but its mechanism of action as a regulator remains subject to speculation.

Annexin II
Annexin II is another example of a Ca^{2+} and phospholipid binding protein postulated to be involved in exocytotic regulation. The first suggestion of a regulatory function for annexin II came with the finding that it can aggregate adrenal chromaffin cell granules at Ca^{2+} concentrations similar to those required for the stimulation of secretion from permeabilised cells (Drust and Creutz, 1988), and it was later found to enhance secretion from run-down chromaffin cells (Ali et al., 1989; Ali and Burgoyne, 1990; Sarafian et al., 1991). This latter activity was proposed to involve phosphorylation of annexin II by PKC (Sarafian et al., 1991). However, recent studies employing overexpression and aggregation of annexin II revealed no significant changes in the levels of Ca^{2+}-dependent secretion from PC12 cells, provoking the conclusion that annexin II might not be essential (Graham et al., 1997). Again, there are as yet no data available regarding a role for annexin II in mast cell secretion.

14-3-3 proteins
These adapter proteins are understood to be involved in signal transduction mechanisms (reviewed in (Burbelo and Hall, 1995)), although how they exert their effects remains subject to much speculation. They are present in adrenal chromaffin cells (Roth et al., 1994) and were assigned a role as exocytotic regulators in these cells since they were found to support Ca^{2+}-dependent secretion from run-down cells (Morgan and Burgoyne, 1992a; Wu et al., 1992). In addition, anti-(14-3-3 protein) antibodies were found to inhibit chromaffin cell secretion (Wu et al., 1992). The enhancing activity of the protein was shown to synergise with that of PKC, but the 14-3-3 proteins do not appear to substrates for phosphorylation. Rather, it was proposed that the synergy arises from the phosphorylation by PKC, of another key protein involved in secretory regulation.
by the 14-3-3 protein (Morgan and Burgoyne, 1992b). It was later found that 14-3-3 proteins are active in the priming phase of exocytosis, but not in the triggering phase. Nor do they appear to play any part in mediating changes in the levels of PIP$_2$ (Chamberlain et al., 1995), previously shown to be important during priming (Hay and Martin, 1993; Hay et al., 1995). 14-3-3 proteins have been demonstrated to facilitate cytoskeletal reorganisations, consistent with a role in priming where they mediate such changes, in order to allow granule access to the plasma membrane (Roth and Burgoyne, 1995).

**Calmodulin (CaM)**
This Ca$^{2+}$-binding protein appears to be essential for exocytosis in *Paramecium tetraurelia* (Kerboeuf et al., 1993) and has been proposed to play a regulatory role in secretion from many cell types. Unfortunately, much of this work has been based on the use CaM inhibitors (such as phenothiazines and W7) which have subsequently been found to be rather non-specific in their effects. Work carried out using protein itself (instead of inhibitors) has lead to the proposal that CaM is involved in adrenal chromaffin cell secretion (Okabe et al., 1992), and it has recently been shown that CaM stimulates the triggering stage of chromaffin cell exocytosis (Chamberlain et al., 1995), making it another candidate for the “high affinity” Ca$^{2+}$ sensor.

There have been some suggestions that the exocytotic mechanism of mast cells is also under regulation of CaM but these conclusions, unfortunately, were once again based on the use of rather non-specific inhibitors (e.g. (Douglas and Nemeth, 1982; Gigl et al., 1987)). A more recent investigation using (more) specific peptide inhibitors, now reveals that secretion occurs even when CaM is selectively inhibited (Sullivan & Koffer, manuscript submitted).

**Rabphilin-3A**
This protein is another candidate for the Ca$^{2+}$ sensor involved in synaptic vesicle exocytosis. It has also been shown to bind to Rab3A (Yamaguchi et al., 1993) and therefore it is discussed in more detail under “GTP-binding proteins in the regulation of exocytosis - Rab3”.
**Doc2**

Doc2 is a Ca\(^{2+}\) and phospholipid binding protein, initially identified in brain, and found to be enriched in synaptic vesicles (Orita et al., 1995). It has recently been found to be present in, and to enhance secretion from PC12 cells (Orita et al., 1996) and it too has been put forward as another possible high affinity Ca\(^{2+}\)-sensor for secretion, although its Ca\(^{2+}\) affinity is as yet unknown. No data exist regarding a role for this protein in any other secretory cell types, including mast cells.

**Cysteine string proteins (Csps)**

These proteins are cysteine rich proteins initially found to be localised to synaptic vesicles (Mastrogiacomo et al., 1994), and are proposed to be involved in neurotransmitter secretion (Zinsmaier et al., 1994; Umbach et al., 1994).

Subsequent findings that Csps are expressed more widely, in non-neuronal as well as neuronal tissues, suggests a role for Csps in general fusion events rather than just in regulated ones (Chamberlain and Burgoyne, 1996). The demonstration that they are associated with adrenal chromaffin cell granules (Chamberlain et al., 1996) certainly points to a role in regulated fusion, though this has yet to be clarified. Again, there are as yet no available data regarding a role for these proteins in mast cell fusion events.

**Cytoskeleton and exocytosis**

Secretory cells have a network of actin filaments in their cortical regions, and this is thought to act as a barrier to exocytosis preventing non-regulated fusion of secretory granules with the membrane. This barrier is then removed upon stimulation of exocytosis, allowing granules to move towards and fuse with the plasma membrane. Such an idea is supported by the findings that, for example in adrenal chromaffin cells, only a few granules are apparent near the plasma membrane in resting cells, yet when stimulated to secrete, disassembly of the actin network is apparent in discrete cortical regions, and the contents of multiple granules are released at these sites of disassembly (reviewed in (Trifaro and Vitale, 1993)).

In mast cells in particular, the role of the cytoskeleton in secretion has been the subject of controversy. Mast cells certainly do exhibit a cortical filamentous actin
network and this is retained after permeabilisation with streptolysin-O (Koffer et al., 1990). Disassembly of this network accompanies stimulated secretion (Norman et al., 1994), and studies employing actin stabilising and destabilising compounds also indicate that cytoskeletal rearrangements correlate with exocytosis from mast cells (Koffer et al., 1990) and RBL-2H3 cells (Liu et al., 1987). However, other studies using the same classes of compounds have cast doubt on the idea that such rearrangements constitute an essential step in the sequence of events leading to exocytosis from mast cells. In these studies, the extent of secretion from mast cells is completely unaffected by facilitating or blocking cytoskeletal rearrangements in this manner (Nemeth and Douglas, 1978; Norman et al., 1996) (Sullivan & Koffer, manuscript submitted). It seems probable that cytoskeletal changes normally take place alongside, but do not dictate events leading to secretion from these cells.

**GTP-binding proteins in the regulation of exocytosis**

As already mentioned, the GTP-binding protein(s) regulating exocytosis, $G_e$, was originally described in neutrophils (Barrowman et al., 1986; Gomperts et al., 1987a), and since then, various GTP-binding proteins of both the heterotrimeric and monomeric classes have been postulated to fulfil such a role (Gomperts, 1990). In this section, the candidates for $G_e$ put forward so far will be discussed.

**Heterotrimeric G-Proteins**

These proteins consist of $\alpha$, $\beta$, and $\gamma$ subunits, and are attached to the cytoplasmic side of the plasma membrane in close association with cell surface receptors. Stimulation of the receptor activates the G-protein by promoting GDP dissociation from the $\alpha$ subunit, allowing GTP to bind in its place. According to current ideas, G-protein activation leads to dissociation of the $\alpha\beta\gamma$ complex from the receptor, separation of the $\alpha$ subunit from the $\beta\gamma$ subunits, whereupon the free $\alpha$ and $\beta\gamma$ subunits stimulate their downstream effectors. GTP hydrolysis by the intrinsic GTPase of the $\alpha$ subunit determines inactivation, resulting in dissociation from the effector and reassociation with $\beta\gamma$ subunits (Bourne et al., 1990). Recent experiments in our laboratory indicate that $\beta\gamma$ subunits are capable of enhancing secretion from run-down mast cells (Pinxteren et al., 1997), though an activating
guanine nucleotide is still required. The stimulus delivered by the βγ subunits cannot therefore comprise a sufficient signal. However, the inactive form of αi3 (αi3.GDP which would be expected to bind to βγ subunits) causes inhibition of secretion stimulated by GTPγS and this can be taken as another pointer for a regulatory role of βγ subunits. The activated form of αi3 (αi3.GDP.[AlF4]') is without effect.

Direct evidence for the involvement of heterotrimeric GTP-binding proteins in secretory regulation came with the finding that Pertussis toxin, which has been shown to inactivate Gi and Go heterotrimers selectively by ADP-ribosylation of the α subunit (reviewed in (Bimbaumer et al., 1990)), has modulatory effects on the secretory responses of many cell types. For example, treatment with Pertussis toxin causes inhibition of secretion from PC12 cells (Inoue and Kenimer, 1988), neutrophils and HL60 cells (Krause et al., 1985). It inhibits secretion from mast cells stimulated by non-immunological stimuli such as mastoparan and compound 48/80 (Nakamura and Ui, 1985; Nakamura and Ui, 1983; Nakamura and Ui, 1984). It also inhibits secretion from RBL-2H3 cells which have been stably transfected with PAF receptor (Ali et al., 1994), but is without effect on either cell type when stimulated through the IgE pathway. In other cell types, including adrenal chromaffin cells (Tanaka et al., 1987; Brocklehurst and Pollard, 1988; Sontag et al., 1991) and pituitary cells (Cronin et al., 1984) it potentiates secretion.

Additional evidence for a role for heterotrimeric GTP-binding proteins in regulated secretion came with the discovery that secretion can be induced by reagents such as mastoparan and aluminium fluoride which are known to cause selective activation of heterotrimeric GTP-binding proteins (Aridor et al., 1990; Mousli et al., 1990b; Kahn, 1991). Aluminium fluoride has been shown to stimulate secretion from a variety of secretory cell types, including mast cells (Kuza and Kazimierczak, 1982; Sorimachi et al., 1988; Aridor et al., 1993), adrenal chromaffin cells (Vitale et al., 1994b), and pancreatic acini (Matozaki et al., 1988). Mastoparan has been found to cause selective activation of Gi and Gi (over Gs and Go) (Higashijima et al., 1988) due to direct interaction of the secretagogue with the carboxyl terminus of the protein (Weingarten et al., 1990; Mousli et al., 1990a). Since mastoparan can also stimulate exocytosis from many
cell types such as mast cells (Mousli et al., 1989; Mousli et al., 1991), adrenal chromaffin cells (Kumakura et al., 1993), PC12 cells (Murayama et al., 1996), pancreatic β-cells (Jones et al., 1993), RINm5F cells (Hillaire Buys et al., 1992), and platelets (Wheeler-Jones et al., 1992), then heterotrimeric GTP-binding proteins such as G₁ and Gᵢ must be considered as prime candidates for exocytotic regulators in these cells. However, recent studies now indicate that mastoparan may not be quite so specific in its activities when introduced into permeabilised cells. In particular, it appears to promote nucleoside diphosphate kinase-catalysed production of GTP from GDP (Kowluru et al., 1995; Marciniak and Edwardson, 1996), and it also acts to elevate the ionic permeability of phospholipid lipid bilayers through the formation of ion channels (Mellor and Sansom, 1990).

In chromaffin cells, Pertussis toxin substrates of 39-41kDa have been identified in membranous fractions (Toutant et al., 1987; Brocklehurst and Pollard, 1988; Tanaka et al., 1987) and one study identified a 39kDa substrate as the α-subunit of G₀ (Toutant et al., 1987). These studies were extended by the findings that, as already mentioned, Pertussis toxin enhances Ca²⁺-dependent exocytosis from streptolysin-O permeabilised chromaffin cells, and this enhancing effect progressively diminishes with time after permeabilisation due to the leakage of GTP-binding protein α-subunits. It was concluded that an inhibitory, Pertussis toxin sensitive GTP-binding protein acts as a regulator in the late stages of Ca²⁺-dependent secretion in these cells (Sontag et al., 1991). Since mastoparan inhibits Ca²⁺-dependent chromaffin cell secretion and this can be reversed by anti-G₀α (but not anti-Gᵢα) antibodies, G₀ appears likely to be the G-protein mediating inhibition. It was also suggested that the inhibition is mediated by the component of G₀ which associates with granule membranes (Vitale et al., 1993; Toutant et al., 1987).

The neuronal protein GAP-43 has been put forward as a possible "pseudoreceptor" for granule-associated G₀ in chromaffin cells, since this protein can stimulate G₀ and has been shown to inhibit Ca²⁺-dependent exocytosis from these cells. This effect can be reversed by anti-G₀α antibodies or GAP-43 peptides corresponding to the domain mediating the interaction with G₀α and this reversal,
in turn, is overturned by a peptide corresponding to the C-terminus of Go (Vitale et al., 1994a).

The use of mastoparan as a secretagogue for investigating the role of heterotrimers in chromaffin cell exocytotic mechanisms also lead to the discovery that not only can this compound inhibit Mg.ATP-dependent Ca\(^{2+}\)-induced secretion, but it can also stimulate Ca\(^{2+}\)-dependent, Mg.ATP independent secretion. Whilst this mastoparan-induced Mg.ATP-independent secretion appears to be sensitive to Pertussis toxin, the corresponding Mg.ATP-dependent inhibitory effects of mastoparan are resistant (Vitale et al., 1994b) (in contrast to the earlier data which indicated otherwise (Sontag et al., 1991)). These observations imply that the two stages of chromaffin cell secretion i.e. Mg.ATP-dependent “priming” and Mg.ATP-independent “triggering” (see under “ATP”) are under the control of different heterotrimeric GTP-binding proteins.

The earlier work postulating granule-associated Go as an inhibitory regulator of chromaffin cell secretion has been supported and extended by similar studies investigating the nature of the heterotrimeric proteins mediating each stage of exocytosis from permeabilised cells (Vitale et al., 1996). The inhibition by mastoparan of Mg.ATP-dependent secretion can be reversed by anti-Go\(\alpha\) antibodies and a peptide corresponding to the C-terminus of Go\(\alpha\). Additional evidence indicates that Go is preferentially associated with chromaffin granule membranes. Mastoparan-mediated increases in Ca\(^{2+}\)-induced, Mg.ATP-independent secretion appear to be inhibited by anti-Gia\(3\) antibodies or a peptide corresponding to the C-terminus of Gia\(3\), and there is evidence that Gia\(3\) is preferentially associated with the plasma membrane. Taken together, these results suggest that in chromaffin cells, Go regulates the initial Ca\(^{2+}\)-induced, Mg.ATP-dependent priming step, and Gia\(3\) regulates the subsequent Ca\(^{2+}\)-induced, Mg.ATP-independent triggering step of exocytosis. The hypothesis that a granule-associated Go protein controls priming is supported by data which has emerged since, indicating that secretion induced by nicotine or by depolarisation (due to high K\(^+\)) from single chromaffin cells is modified in a similar manner by micro-injection of mastoparan or anti-Gia\(3\)/Go\(\alpha\) antibodies (Vitale et al., 1997). In addition, secretion induced by the same stimuli was shown to be inhibited by micro-injection of a GAP-43 peptide.
corresponding to the domain interacting with \( G_o \). It has thus been proposed that GAP-43 (or similar) regulates priming of chromaffin cell exocytosis by activating \( G_o \).

Although there is no evidence for the involvement of \( G_o \), there do exist data supporting a role for \( G_{i3} \) in the regulation of secretion following stimulation by non-immunological ligands such as compound 48/80 and mastoparan. Aridor et al have demonstrated the presence of \( G_{ai2} \) and \( G_{ai3} \) and showed that anti-\( G_{ai2} \) antibodies localise exclusively to the plasma membrane, whilst anti-\( G_{ai3} \) antibodies bind to both plasma and Golgi. The introduction of either a peptide corresponding to the C-terminus of \( G_{ai3} \) or specific antibodies against the same peptide inhibits mast cell secretion induced by compound 48/80 (Aridor et al., 1993). The equivalent \( G_{ai2} \) reagents are without effect. Since brefeldin A (which disrupts the organisation of Golgi membranes) is without effect on 48/80-induced mast cell secretion it is likely that the inhibition of secretion by the \( G_{ai3} \) derived peptides and antibody is mediated by the plasma membrane-associated pool of \( G_{i3} \) (Aridor et al., 1993). Although there are no data, so far, to support the existence of distinct priming and triggering stages in the sequence of events leading to exocytosis in mast cells, we should not lose sight of the possibility that \( G_{i3} \) might fulfil a similar role to that proposed for chromaffin and PC12 cells.

**Monomeric or small GTP-binding proteins (Ras-related GTPases)**

The Ras-related GTPases have been the subject of intense research over the past decade and it has become increasingly clear that these proteins control a wide variety of cellular processes. Similar to the heterotrimeric GTPases, each protein cycles between an “active”, GTP-bound state and an “inactive” GDP-bound state. This cycling is influenced by regulatory proteins which promote activation or deactivation, or protect the protein from switching between each state. These proteins are called GEF (guanine nucleotide exchange factor), GAP (GTPase activating protein), and GDI (guanine nucleotide dissociation inhibitor), respectively.

The Ras-related superfamily can be divided into subfamilies which include Ras, Rho, Rab, Arf, Rap, Ral and Ran. Each of these families has been postulated to be involved in the regulation of specific cellular processes (although it is becoming
increasingly clear that some subfamilies can regulate more than one process). In addition, each subfamily differs slightly in the mechanisms whereby its members exert their effects, and also differs in their regulation, although the general rules outlined above apply in most cases. Four of these subfamilies (Ras, Rab, Arf and Rho) have been implicated in the regulation of secretion, and these will be the focus of this discussion.

All Ras-related proteins are post-translationally modified. At the point of synthesis, the proteins of each subfamily possess a sequence which is a signal for post-translational modification. In the Ras, Rab and Rho proteins this motif (-CAAX) is present at the C-terminus whilst in Arf proteins it is expressed at the N-terminus. The modified form of Ras is farnesylated, whilst both Rho and Rab are geranylgeranylated, and ARF is myristoylated (Boguski and McCormick, 1993; Kahn et al., 1988). For Ras, Rab and Rho proteins, prenylation is followed by proteolytic cleavage of the terminal three or two amino acids, where relevant, and carboxymethylation of the isoprenyl-modified cysteine (Bokoch and Der, 1993; Boguski and McCormick, 1993). Exceptions to these general rules include RhoB, which is modified by farnesyland/or geranylgeranyl groups (Adamson et al., 1992), RhoE, which is farnesylated (Foster et al., 1996), and Rab proteins with two cysteines at the extreme C-terminus which do not undergo carboxymethylation (Smeland et al., 1994).

These post-translational modifications play a crucial role in the activity of Ras-related proteins, since all the proteins require membrane localisation in order to exert their biological effects (Bokoch and Der, 1993; Boguski and McCormick, 1993). However, whilst Ras remains membrane-bound regardless of its state of activation, the other Ras-related proteins cycle in and out of their target membranes when active or inactive, respectively. Arf is able to move in and out of the membrane unaided because of the presence of a hydrophobic pocket in its structure which serves to conceal the lipid component as it moves from the membrane to the cytosol. In contrast, Rab and Rho proteins are assisted in their movements by cytosolic GDI proteins, specific for each subfamily, and which can only interact with lipid-modified protein (Beranger et al., 1994; Soldati et al., 1993; Hancock and Hall, 1993; Chuang et al., 1993; Hart et al., 1992; Leonard et al., 1992). The function of these GDI proteins is to facilitate the movement of Rab
and Rho proteins between cytosol and membrane, and also to target the GTPase to the specific membrane where it exerts its action. They do this by effectively "masking" the lipid moiety in these proteins, making them more hydrophilic so they can enter the cytosol so long as they remain part of the protein-GDI complex. In unstimulated cells, Rho proteins are normally found to be present in the cytosol, complexed to their specific GDI (Regazzi et al., 1992; Kuroda et al., 1992), while Rab proteins, which play an essential role in the constitutive secretory pathway (with the exception of Rab3 which is discussed in detail later), cycle continuously between active and inactive forms and are to be found distributed in both cytosolic and membrane fractions. Upon stimulation, it is understood that the Rho or Rab protein translocates to the plasma membrane in association with GDI, where dissociation of the protein-GDI complex occurs and the protein becomes membrane-associated. After GTP hydrolysis, the GDI-protein complex reforms, removing the GDP-bound, inactive protein from the membrane (Hall, 1992; Pfeffer, 1992).

The interconversion of Ras-related proteins between GDP and GTP bound states is also under the control of specific GEFs and GAPs. GEFs promote the conversion of the protein from its GDP-bound to its GTP-bound form by catalysing the dissociation of GDP. GEFs specific for Ras, Rab, ARF, and Rho have been identified (Boguski and McCormick, 1993; Banin et al., 1996; Tsai et al., 1996; Chardin et al., 1996). In addition, GAPs specific for all these proteins have also been identified (Boguski and McCormick, 1993; Lamarche and Hall, 1994; Burstein and Macara, 1992; Strom et al., 1993; Xiao et al., 1997; Makler et al., 1995; Cukierman et al., 1995; Poon et al., 1996). These promote the inactivation of GTP-bound protein by stimulating the protein's intrinsic GTPase activity, which is very low in the absence of GAP. However, it has also been suggested that GAPs might also act as effector proteins (e.g. (Pomerance et al., 1992; McGlade et al., 1993)) Not only does RasGAP bind to the Ras effector domain (reviewed in (McCormick, 1989)) but its full sequence reveals other recognisable inserts including two SH2 domains, one SH3 domain, a PH (pleckstrin-homology) domain, proline-rich sequences and a motif (CALB) which is expressed in proteins (such as PLA$_2$) which bind to phospholipids when the concentration of Ca$^{2+}$ is elevated. However, it appears that most of the evidence for an effector function is so far suggestive rather than firm.
Ras
Bar-Sagi & Gomperts (Bar-Sagi and Gomperts, 1988) demonstrated that micro-injection of oncogenic Ras (but not the proto-oncogenic form) into mast cells induces secretion in the absence of an external stimulus. This activity was found to occur only in the presence of external Ca\(^{2+}\). However, secretion only commences 2.5-4 hours after introduction of the protein. This indicates that the stimulatory effect of Ras is unlikely to be direct, since exocytosis is an acute event, occurring rapidly following application of a stimulus. In addition, evidence was presented suggesting that only low levels of Ras protein are found in mast cells. It therefore appears from these data that Ras is not directly involved in the regulation of exocytosis from mast cells, but is able to exert indirect effects on signalling pathways regulating exocytosis when present in its activated form for prolonged periods.

Rab3
The Rab family of small GTP-binding proteins, of which there are known to be at least 30 subtypes expressed in animal cells, are involved in the process of vesicular trafficking (reviewed in (Pfeffer, 1994)). Each Rab protein is found localised to distinct compartments of the exo- or endocytic pathways, leading to the idea that Rab proteins act to ensure the specificity of membrane-membrane interactions.

One member of this family, Rab3 (of which four isoforms have been identified: Rabs 3A, 3B, 3C, and 3D), appears to be restricted to the membranes of organelles involved in the process of regulated exocytosis. For example, Rabs 3A and 3C are present on synaptic vesicles (Fischer von Mollard et al., 1990; Fischer von Mollard et al., 1994; Matteoli et al., 1991). Secretory granules of pancreatic acinar cells are reported as containing Rabs 3D (Valentijn et al., 1996) and 3A or 3B (the antibody used in this latter study did not distinguish between the two forms) (Jena et al., 1994). In chromaffin cells, Rab3A may be expressed on chromaffin granules (Darchen et al., 1990; Darchen et al., 1995), though another report claims that Rab3 is localised to synaptic-like microvesicles (Fischer von Mollard et al., 1990). A survey employing Western blotting coupled with ECL detection failed to find Rab3 proteins in cellular extracts and subcellular fractions of guinea pig...
eosinophils though other Rab proteins were clearly evident (Lacy et al., 1995). In general however, the restricted expression of Rab3 GTPases on regulated exocytotic organelles, coupled with the finding that this protein translocates to the plasma membrane (Matteoli et al., 1991), and dissociates from synaptic vesicles during or after exocytosis (Fischer von Mollard et al., 1991; Fischer von Mollard et al., 1994), has suggested that Rab3 may be involved in the control of regulated secretion.

Much work investigating this possibility has been based on the use of peptides corresponding to the effector domain of Rab3 to probe for its involvement in the secretory process. The effector domain has been defined by analogy with the corresponding sequence of Ras and comprises roughly the sequence of amino acids between residues 37 and 44 (Pfeffer, 1992). Synthetic peptides based on this sequence had first been used in the investigation of constitutive secretory mechanisms, where they inhibit vesicular transport (Plutner et al., 1990). On the other hand, when introduced into the cytosol of many secretory cell types, including pancreatic acini (Padfield et al., 1992; Edwardson et al., 1993), chromaffin cells (Senyshyn et al., 1992), mast cells (Oberhauser et al., 1992), and HIT-T15 cells (Li et al., 1993), effector domain peptides were found to enhance secretion and it was concluded that Rab3 acts as a regulator for exocytosis. However, when these same peptides are introduced into pituitary cells they cause inhibition (Davidson et al., 1993). Yet another report contradicts the earlier proposal concerning the effect of these peptides as regulators of secretion from chromaffin cells (Morgan and Burgoyne, 1993). Further doubt concerning their action as regulators came with the findings that the peptides have multiple effects, stimulating IP₃ accumulation when applied to permeabilised pancreatic acini (Piiper et al., 1993), and suppressing cAMP levels and inducing Ca²⁺ transients in mast cells (Law et al., 1993). It is now generally accepted that the Rab3 effector domain peptides are rather non-specific in their effects. This conclusion was confirmed with the demonstration that a random (scrambled) peptide based on the amino acids that comprise the Rab3 effector domain retains its capability of enhancing secretion from permeabilised pancreatic acinar cells (MacLean et al., 1993). In addition, monoclonal antibodies raised against Rab3A and which bind to the membrane of pancreatic zymogen granules have no effect on secretion. In conclusion, it now seems that the initial excitement based on the use of peptides
mimicking the effector domain, and which pointed to Rab3 as a regulator of secretion, possibly the elusive Ge, was somewhat misplaced.

In spite of this, alternative approaches have provided some support for the idea that the Rab3 GTPases act as regulators of exocytotic secretion. Introduction by whole cell patch clamp of antisense oligonucleotides which suppress Rab3B expression in pituitary cells is reported to inhibit exocytosis (Lledo et al., 1993). In contrast, overexpression of Rab3A, or expression of a mutant form (Rab3A-Q81L) which has reduced intrinsic GTPase activity and which is relatively insensitive to RabGAP (by analogy to Ras), inhibits secretion from chromaffin cells stimulated by nicotinic agonists (Holz et al., 1994). Ca^2+-induced secretion from permeabilised cells is also inhibited when this mutant protein is highly expressed. In addition, expression of the mutant Rab3A-N135I, thought to be another dominant active form (since it may be predominantly GTP-bound within the cell by analogy with the equivalent Ras mutant), causes limited inhibition, whilst expression of Rab3A-T36N, which is a dominant negative form (since it has ten fold higher affinity for Rab3A-GEF than wild type Rab3A, and does not detectably bind GTP), is without effect. From these results it has been concluded that the activated, GTP-bound form of Rab3A is inhibitory to exocytosis, and this inhibition must be overcome for secretion to occur. It has also been suggested that Rab3A functions as part of a pre-fusion complex with secretory vesicles (Holz et al., 1994). Similarly, secretion from chromaffin and PC12 cells micro-injected with Myc-tagged Rab3A-Q81L or Myc-tagged Rab3A-N135I is suppressed (Johannes et al., 1994). A similar mechanism of action for Rab3A as an inhibitory regulator of exocytosis has been proposed. A third report, while offering support for the idea of such an inhibitory role of Rab3A in PC12 cells, indicates that overexpression of Rab3B-N135I enhances the level of secretion (Weber et al., 1996). However, evidence suggests that Rab3B is not normally expressed in these cells (Weber et al., 1994), so the significance of these data is not clear.

Similarly, there must also be some doubt concerning the significance of the finding that expression of Rab3A-N135I inhibits secretion induced by IgE-R crosslinking in RBL-2H3 cells, since no evidence could be found of normal expression of any wild type Rab3 isoform in these cells. Furthermore, since the
block in secretion can be overcome by stimulating permeabilised cells with GTPyS, it follows that the Rab acts upstream of another GTP-binding protein more immediately associated with the regulation of membrane fusion (Smith et al., 1997). However, if one accepts that these data do reflect the effects of a mutant form of a native protein, then these too support a role for Rab3A as a negative regulator of secretion, albeit in a rather different role than that previously proposed. Transgenic mice possessing a mutant Rab3A gene appear to be generally unaffected by the mutation, but synaptic depression is found to be prolonged following repetitive stimulation. The conclusion is that Rab3A is involved in recruitment or priming of vesicles for secretion (Geppert et al., 1994a).

There is growing evidence supporting a role for Rab3 in the formation of SNARE complexes which occurs when secretory vesicles dock onto the plasma membrane before exocytosis. In ER to Golgi transport, the yeast Rab homologue Ypt1p is required for assembly of SNARE complexes (although it apparently does not constitute a part of the complex itself) (Sogaard et al., 1994), and has in addition been postulated to confer specificity to the fusion reaction by selectively activating individual v-SNAREs (Lian et al., 1994). In the control of regulated exocytosis, there is evidence that Rab3 may fulfil a similar role, and that GTP hydrolysis by Rab3 is essential for secretion to proceed. As might be expected from the general “cycling” model underlying small GTPase function, in isolated nerve terminals, synaptic vesicle-bound Rab3A is predominantly in the GTP-bound form, whilst cytosolic Rab3A is GDP-bound and associated with RabGDI. When nerve terminals are stimulated to secrete there is an increase in the GDP/GTP ratio of Rab3-bound nucleotide indicating an enhancement of Rab3 GTPase activity. Consistent with this, approximately half of the Rab3A in fractions containing plasma membrane-synaptic vesicle complexes is in the GDP-bound form, and half is in the GTP-bound form (Stahl et al., 1994). Further, GTPase deficient Rab3 microinjected into Aplysia neurons inhibits secretion but fails to do so when it is also modified to prevent its association with the membrane (Johannes et al., 1996). The onset of inhibition of exocytosis by tetanus and botulinum A toxins is delayed by the presence of the GTPase deficient mutant, and since these toxins fail to cleave their substrates (VAMP/synaptobrevin and SNAP-25, respectively) when they are associated together as part of the SNARE complex (Pellegrini et al.,
it is likely that the Rab3-GTP has a stabilising effect on the assembled components (Johannes et al., 1996). The demonstration that Rab3A plus guanine nucleotides delays secretion when applied by patch pipette to chromaffin cells is also consistent with this idea (Lin et al., 1996). Taken together with the earlier findings that Rab dissociates from secretory vesicles following exocytosis (Fischer von Mollard et al., 1991; Fischer von Mollard et al., 1994), these data indicate that Rab3 controls the formation or stability of the fusion complex in regulated exocytosis.

Exactly how Rab3 performs such a function remains a subject of speculation, but a clue is provided by the finding that the GTPase-deficient Rab3 mutant fails to inhibit exocytosis when it also possesses a mutation in its effector domain (Johannes et al., 1996). The most widely studied effector of Rab3 is the Ca^{2+}/phospholipid binding protein Rabphilin 3A. This is associated with adrenal chromaffin cell granule membranes (Yamaguchi et al., 1993) and is found to support secretion (Chung et al., 1995). Rabphilin 3A may also be involved in the cortical granule release reaction of mouse eggs (Masumoto et al., 1996) and also in Ca^{2+}-dependent exocytosis from PC12 cells (Komuro et al., 1996a) in which the protein is concentrated at sites of membrane fusion (Wada et al., 1994). There is some controversy about whether it is expressed in insulin secreting cells (Regazzi et al., 1996; Inagaki et al., 1994), and so far there is no indication of a direct effect of Rabphilin 3A on insulin secretion.

Rabphilin 3A must certainly be considered as a prime candidate for an effector for Rab3 in the regulation of exocytosis. Like Rab3, Rabphilin 3A is associated with synaptic vesicles (Shirataki et al., 1994; Stahl et al., 1996; Senbonmatsu et al., 1996), but there is disagreement concerning the mechanism by which it binds. Initially, Rabphilin 3A was thought to bind directly (Shirataki et al., 1994), but more recently it was reported to bind to Rab3 (and hence, to synaptic vesicles) in a GTP-dependent manner and then to dissociate along with Rab3, in a manner requiring Ca^{2+} and membrane fusion (i.e. exocytosis) to occur (Stahl et al., 1996). It has also been suggested that the binding of Rabphilin 3A to Rab3A serves to target the protein to the synaptic vesicle membrane (McKiernan et al., 1996). In addition, Rabphilin 3A inhibits RabGAP activity (Kishida et al., 1993) and so it can prolong the period during which Rab and Rabphilin 3A remain associated.
This is certainly consistent with a role for Rab3 (along with its effector Rabphilin 3A) as a negative regulator of secretion. Rab3 may act to stabilise the fusion complex, preventing exocytosis occurring whilst it remains associated together with Rabphilin 3A on the synaptic vesicle membrane.

On the basis of the available data a scheme summarising the role of the GTPase cycle of Rab3 has been presented (Johannes et al., 1996). GTP-bound Rab3, localised to the synaptic vesicle membrane recruits, or binds, its effector Rabphilin 3A. This promotes vesicle docking at the plasma membrane, ready for rapid exocytosis after receipt of an appropriate stimulus. Inactivation of the Rab protein is prevented by the inhibitory action of Rabphilin 3A on RabGAP and this holds the vesicle in its docked position. When a Ca\(^{2+}\) stimulus is received, GAP-stimulated GTP hydrolysis occurs, Rab3 and Rabphilin 3A dissociate from the synaptic vesicle and exocytosis occurs (Sudhof, 1997). The GDP-bound Rab3 is escorted back to free, undocked synaptic vesicles by RabGDI. In order for Rab3 to reassociate with vesicles, nucleotide exchange must occur, and this is catalysed by a GEF. Although RabGDI can interact with many different Rab proteins (Araki et al., 1990), the specificity of the attachment of activated Rab3 to synaptic vesicles is probably ensured by the presence of a GEF specific for Rab3 (Burstein and Macara, 1992; Wada et al., 1997). Similarly, RabGAPs specific for Rab3 have also been identified (Burstein and Macara, 1992; Fukui et al., 1997). This may ensure that release of synaptic vesicles cannot not occur in a non-regulated fashion.

Exactly how fusion proceeds following GTP hydrolysis and dissociation of Rab3 and Rabphilin 3A remains far from understood, and only one possible explanation has been put forward so far. The demonstration that Rabphilin 3A can interact with \(\alpha\)-actinin, which cross-links actin filaments into bundles, has led to the suggestion that following GAP-stimulated GTP hydrolysis on Rab3A, the dissociated Rabphilin 3A is available to interact with \(\alpha\)-actinin. This causes bundling of actin filaments which, by removing the proposed "cytoskeletal barrier" to exocytosis, may then allow membrane fusion to occur (Kato et al., 1996). Rabphilin 3A has also been shown to bind to \(\beta\)-adducin, another protein involved in cytoskeletal organisation, although the role of this protein is unclear.
(Miyazaki et al., 1994). Hence if Rabphilin 3A is indeed the Rab3A effector regulating exocytosis, then its effects may be mediated through interactions with the cytoskeleton. However, in those systems where secretion can occur in the absence of cytoskeletal changes, Rabphilin 3A may be either redundant or it may exert its effects via an alternative, as yet unidentified pathway.

**ADP-ribosylation factor (ARF)**

ARF regulates a range of vesicle transport and fusion steps along the secretory and endocytic pathways (Donaldson and Klausner, 1994), and at least six isoforms of ARF have been isolated. ARF can also regulate phospholipase D (PLD), causing hydrolysis of phosphatidylcholine (PC) to produce phosphatidic acid (PA) and choline (Brown et al., 1993; Cockcroft et al., 1994). PLD activation does not always occur in response to addition of ARF, but it has been suggested that this reflects the existence of different isozymes and/or assay conditions in *in vitro* experiments, implying isozyme specificity in the PLD/ARF interaction (Malcolm et al., 1994).

A possible role for ARF as a regulator of secretion first emerged with the demonstration that the stimulation of permeabilised insulin-secreting RINm5F cells induces concurrent re-distribution of ARF to Golgi and plasma membranes, though no direct evidence was produced to show that ARF is a regulator of the secretory response (Regazzi et al., 1991). Further evidence came with the finding that a synthetic peptide corresponding to the N-terminus of ARF1 can inhibit secretion from run-down adrenal chromaffin cells (Morgan and Burgoyne, 1993), but these data were questioned when the peptide was found to be non-specific, causing inhibition of PLCβ (considered to be ARF-independent) as well as PLD and exocytosis in run-down HL60 cells (Fensome et al., 1994). Later studies (discussed in detail later in this section) have indicated that PLC activation is also, though indirectly, dependent on ARF, since ARF1 stimulates synthesis of the its substrate PIP2 (Fensome et al., 1996). Thus the earlier reports (Morgan and Burgoyne, 1993) implicating ARF in exocytosis from chromaffin cells by use of the ARF peptide remain valid, although it seems that its role is more likely to be that of a housekeeper than a regulator.
There are additional pieces of (mostly indirect) evidence implicating ARF in the regulation of secretion. ARF, presumably by activating PLD to produce PA, which activates PIP-5 kinase (PIP-5K), promotes PIP$_2$ synthesis (Fensome et al., 1996; Martin et al., 1996). This enzyme has already been shown to be involved in the priming step of chromaffin cell exocytosis, as already discussed (see under "ATP"). The importance of PIP$_2$ is underlined by the finding that the addition of PI-specific PLC blocks secretion from permeabilised chromaffin cells (Eberhard et al., 1990), and semi-intact PC12 cells (Hay et al., 1995). Secretion from semi-intact PC12 cells can also be blocked by the addition of antibodies against PIP$_2$ (Hay et al., 1995). Also, it has been demonstrated that PIP$_2$ stimulates ARF-GAP activity to promote inactivation of ARF, and there appears to be co-ordinated regulation of ARF and PLD through modulation of ARF-GAP activity. The PLD substrate, phosphatidylcholine (PC) appears to inhibit ARF—GAP activity, and this can be reversed by the PLD product, PA, which stimulates ARF—GAP (Randazzo and Kahn, 1994).

All this, taken together with the previous findings concerning the involvement of ARF in the regulation of exocytosis, has lead to the following hypothesis. PA, produced by PLD as a result of stimulation by activated, GTP-bound ARF, activates PIP-5K to produce PIP$_2$. This then further activates PLD in a positive feedback loop. PIP$_2$ (and of course, PA), stimulates the conversion of ARF to its GDP-bound, inactive form (by activating ARF-GAP). This promotes the dissociation of ARF from the vesicles and vesicle fusion with the plasma membrane ensues (Loijens et al., 1996).

The most direct evidence for such a role for ARF in the regulation of secretion has come with the finding that ARF1 can restore secretory activity to run-down HL60 cells (Fensome et al., 1996). Some further support is found in the demonstration that ARF6 is associated with chromaffin granules and that when activated, it dissociates. In addition, a myristoylated peptide corresponding to the N-terminal domain of ARF6 inhibits Ca$^{2+}$-induced secretion from streptolysin-O permeabilised chromaffin cells (Galas et al., 1997). However, the situation regarding the stimulation of PLD may not be as simple as presented above, since its activity appears to be modulated by other cytosolic factors besides ARF such as Rho (Bowman et al., 1993; Malcolm et al., 1994) (although a direct stimulation
has been disputed (Martin et al., 1996) and there exist data which suggest any Rho effects on PLD may be mediated indirectly via modulatory effects on PIP-5K - see later, under "Rho proteins"). These additional factors, some of which have yet to be fully characterised and identified, may also constitute part of the overall mechanism (Singer et al., 1995).

The scheme presented above can also accommodate a role for phosphatidylinositol transfer protein (PI-TP), which, like PIP-5K, has been demonstrated to be involved in the priming step of chromaffin cell secretion (Hay and Martin, 1993), and which enhances secretion from run-down HL60 cells (Fensome et al., 1996). It is conceivable that PI-TP delivers the substrate (PI) required for PIP production by PI-4K, and this PIP is in turn transferred by PI-TP to PIP-5K leading to the production of PIP₂ (Cunningham et al., 1995). Both this and the ARF-stimulated mechanism for PIP₂ synthesis in the regulation of exocytosis may occur alongside each other in a complementary fashion, or alternatively, one may predominate over the other. The actual mechanisms underlying the production of PIP₂ and its role in secretory regulation await further clarification. For the moment, the postulated role of ARF as a regulator of secretion remains controversial.

*Rho family proteins*

The Rho family of Ras-related small GTP-binding proteins comprises Rho (A, B, C), Rac (1 & 2), Cdc42, Rho E, Rho G, and TC10. Although the available data are somewhat limited, it has become apparent that some of these proteins may act as regulators of exocytosis. Constitutively active mutants of RhoA and Rac1 have been found to enhance stimulated secretion from permeabilised, washed mast cells, whilst incubation with a dominant negative Rac1 mutant inhibits secretion. Incubation of these cells with *Clostridium botulinum* C3 ADP-ribosyltransferase (C3 transferase), considered to cause selective ADP-ribosylation of Rho was found to inhibit secretion. These observations have provoked the suggestion that both Rac and Rho are involved in the regulation of secretion from these cells (Price et al., 1995). However, C3 transferase treatment appears to have no effect on secretion from RBL-2H3 cells (Prepens et al., 1996), and nor does it prevent exocytosis when injected (together with NAD⁺) into mast cells in the whole-cell patch-clamp configuration (Mariot and Tatham, unpublished observations). Under
the conditions in which it does cause inhibition (Price et al., 1995), it thus seems likely that this is due to modification of other GTPases of the Rho family by this enzyme.

Another study has provided supporting evidence for a role for Rho and Rac using PC12 cells. Overexpression of RhoGDI inhibits K⁺-induced, Ca²⁺-dependent secretion, and this can be restored by co-expression of constitutively active mutants of RhoA or Rac1, but not Cdc42 (Komuro et al., 1996b). In addition, the overexpression of RhoGDI prevents the cytoskeletal changes which characteristically accompany secretion in these cells. Overexpression of RhoA or Rac1 alone, however, is insufficient to cause enhancement of secretion, leading to the conclusion that all three proteins, namely RhoA, Rac1, and RhoGDI, are involved in the regulation of Ca²⁺-dependent exocytosis in PC12 cells via cytoskeletal reorganisations, but that further components are still necessary for secretion to occur (Komuro et al., 1996b). Additional support for a role for Rho has also come with the demonstration that secretion from permeabilised RBL-2H3 cells stimulated with a variety of stimuli such as Ca²⁺ ± GTPyS, or IgE receptor cross-linking, is inhibited by prior treatment of the cells with C3 transferase (Yonei et al., 1995).

One should consider the possibility that Rac and Rho may mediate their effects on secretion via the cytoskeleton (Komuro et al., 1996b). Certainly, their involvement in cytoskeletal changes is well documented (Hall, 1994; Nobes and Hall, 1995). However, subsequent work has indicated that in mast cells at least, the stimulatory effects of Rac and Rho on secretion may be mediated through other routes since both proteins still enhance secretion even when all cytoskeletal changes have been blocked (Norman et al., 1996).

An alternative mechanism by which Rho may stimulate secretion is by activation of PLD and/or PIP-5K to promote PIP₂ synthesis. There is some dispute over whether Rho truly activates PLD, with some claiming that it can (Bowman et al., 1993; Malcolm et al., 1994; Singer et al., 1995) and others suggesting that Arf, not Rho, is the likely endogenous regulator (Martin et al., 1996). However, the latter report demonstrated that although Rho may not promote PIP₂ synthesis directly by activation of PLD, it can modulate the activity of PIP-5K, raising the possibility
that stimulation of PLD by Rho occurs indirectly by way of PIP-5K activation. There is certainly evidence that Rho does interact with PIP-5K and modulates its activity, but its function in this respect is controversial. Some reports implicate Rho in the activation of PIP-5K (Chong et al., 1994; Zhang et al., 1996) with one demonstrating a direct interaction of the two proteins (Ren et al., 1996) but this claim has been contradicted by others (Tolias et al., 1995), and yet another report offers data showing that Rho even inhibits PIP-5K activity so that removal of Rho acts to stimulate PIP-5K (Martin et al., 1996). So, although it appears that Rho does, or can exert modulatory effects on PIP-5K, the nature of these, even to the point of whether these are positive or negative, remains to be determined. Activation of PIP-5K by Rho could explain its apparent stimulatory effects on secretion. Rac may also exert its effects on secretion by a similar mechanism, since overexpression of Rac1 in Rat1 fibroblasts enhances PLD activity in response to EGF stimulation (but not PDGF or phorbol ester stimulation) (Hess et al., 1997), and Rac1 has also been found to activate PIP-5K (Tolias et al., 1995). There are as yet no other clues regarding the possible mechanism of action of Rac in its postulated role as a secretory regulator.

Data supporting a role for Cdc42 in the regulation of exocytosis are limited. Clostridium difficile toxin B blocks secretion from RBL-2H3 cells induced by antigens (IgE-receptor pathway), carbachol (reacting at a muscarinic cholinergic receptor (m1) introduced by transfection), or mastoparan. In addition, it reduces the extent of secretion in response to the calcium ionophore A23187 (Prepens et al., 1996). The substrates for this toxin have been identified as RhoA and Cdc42, but since C3 transferase (shown to cause selective ADP-ribosylation of RhoA in these experiments) is without effect on secretion, the toxin B substrate involved in the regulation of secretion is likely to be Cdc42. In addition, disruption of the cytoskeleton by C. botulinum C2 toxin or cytochalasin enhances secretion in response to all these secretagogues, yet toxin B can still inhibit secretion, suggesting that this toxin does not act via inhibition of Cdc42-mediated effects on the cytoskeleton (Prepens et al., 1996). An alternative mechanism for regulation of secretion by Cdc42 is based on the observation that addition of guanine nucleotide to permeabilised pancreatic islets or isolated β cells results in membrane association and carboxymethylation of Cdc42, and in addition, inhibitors of
carboxylmethylation suppress nutrient-induced secretion from both cell types (Kowluru et al., 1996). The significance of these data is unclear, however, since it was previously proposed that protein carboxylmethylation is not involved in the acute regulation of exocytosis (Unger et al., 1981).
CHAPTER 2

"MATERIALS AND METHODS"
MATERIALS

Streptolysin-O (SL-O) was obtained from Murex Diagnostics (Dartford, Kent, UK). GTPyS was obtained as a 100mM stock solution of the lithium salt from Boehringer Mannheim (Mannheim, Germany). Deep frozen bovine brains were obtained from Advanced Protein Products (Brierly Hill, West Midlands, UK). All chromatographic and buffer exchange columns were obtained from Pharmacia (Uppsala, Sweden). BCA protein assay kit was obtained from Pierce (Chester, UK). Antibodies against Rac, Rho, G25K and Rho GDI were the gift of Professor Alan Hall. Commercial preparations of Rac1 & Rac2, and Cdc42Hs antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, California, USA). A commercial preparation of anti-RhoGDI antibody was purchased from Transduction Laboratories (Lexington, Kentucky, USA). HRP-coupled anti rabbit IgG was from Pierce (Chester, UK), and HRP-coupled anti mouse IgG was from Biorad (Hemel Hempstead, UK). Enhanced chemiluminescence detection kit was purchased from Amersham (Amersham, UK). Human thrombin was obtained from Sigma as a frozen solution. [32P] GTP was obtained from Amersham. [3H]-GTP and [3H]-GDP solutions were obtained from DuPont NEN (Stevenage, UK).

Transformed E.Coli expressing Rac2-GST and T17N-Rac2-GST were kindly provided by Dr. Janet Smith and Dr. Roberto Solari. Transformed E.Coli expressing Cdc42Hs-GST, C-Myc-Cdc42Hs, and T17N-Cdc42Hs were kindly provided by Professor Alan Hall.

Samples of recombinant Rac1 and Rho used in immunoblotting samples were a gift of Dr Leo Price. HL60 cells and Jurkat cells used in Western blotting studies were the gifts of Dr Emer Cunningham and Mr Ian Gilfillan, respectively.

All other chemicals and reagents were of the highest quality available from standard commercial sources.
METHODS

Secretion Measurements

NB. Two variations of the rundown assay were used in the work presented in this thesis. One was the "rundown time course", which allows monitoring of the rate of rundown of secretion under the chosen conditions (generally with or without test protein). The other was the "fixed rundown time" assay, in which cells were allowed to run down for a predetermined period following permeabilisation, before being stimulated under a variety of conditions. In this way, the effects of putative modulators of secretion from partially rundown mast cells could be determined. It also allowed the direct comparison of the effects of multiple proteins introduced into the cells either separately or in combination. The experimental conditions used for each type of assay were identical.

Solutions

1) Assay buffer: 137mM NaCl, 2.7mM KCl, 1mM MgCl₂, 0.02% NaN₃, 1 μg.ml⁻¹ BSA, 20mM piperazine-N,N'-bis(2-ethane sulfonic acid) [PIPES], pH 6.8.

2) Rundown buffer:

For experiments in Chapter 3 - Assay buffer plus 0.6mM EGTA (Ca²⁺ to <pCa8), 100μM ATP, 3mM creatine phosphate, 0.6 mg.ml⁻¹ creatine kinase.

For experiments in Chapter 4: Assay buffer supplemented with 0.6mM EGTA (Ca²⁺ to <pCa8), and 1mM ATP.

NB. ATP is always present during the rundown. This is because it was found that the rate of rundown of secretory responsiveness of mast cells (to Ca²⁺ and GTPyS) is slowed in the presence of ATP, shown in Figure 2.1. It was important to slow the rundown so that test proteins had enough time to penetrate the cells and exert their effects (if any), before the cells became completely refractory. In this way, any modulatory effects would be easily detected. In addition, for the FOAD II purification assays (Chapter 3) the Rundown buffer was supplemented with an ATP regeneration system as detailed above. This was used to guard against the possibility that ATPases might co-elute from chromatographic columns with FOAD proteins, possibly resulting in masking of FOAD activity simply by
Mast cells were permeabilised by treatment with streptolysin-O at 0°C for 5 min, washed and then brought to 37°C in Rundown buffer (pH 6.8) containing sufficient EGTA (0.1mM) to suppress Ca^{2+} to below pCa8 in the presence (solid symbols) or absence (open symbols) of Mg.ATP (1mM). At various times (indicated) samples were removed and stimulated by transfer to solutions containing Ca.EGTA (3mM) to regulate pCa5 plus GTP\gamma S (100\mu M - stimulated cells only). The incubation was continued for a further 20min, at which time the cells were sedimented by centrifugation and the supernatant assayed for the presence of secreted hexosaminidase. Points indicate % stimulated secretion above control levels (where Ca.EGTA (3mM) to regulate pCa7 was added following rundown) as a percentage of total cellular hexosaminidase. Error bars indicate SEM, n=4, error bars are omitted where too small for inclusion. Similar results were obtained on at least three occasions.

Figure 2.1  Loss of sensitivity (rundown) to stimulation for secretion by mast cells following permeabilisation in the presence and absence of Mg.ATP
increased rundown relative to other column fractions due to the lack of ATP. This precaution was not considered necessary when investigating the effects of purified recombinant proteins (Chapters 4 & 5).

3) **Homogenisation buffer**: Assay buffer with no BSA, plus 100µM ATP, 0.3mM EGTA, 1mM dithiothreitol, 0.1mM phenylmethylsulfonyl fluoride, 1 µg.ml⁻¹ pepstatin A, 1 µg.ml⁻¹ leupeptin.

4) **Replacement buffer**: Assay buffer with no BSA, plus 0.3mM EGTA, 1mM dithiothreitol, 0.1mM phenylmethylsulfonyl fluoride, 1 µg.ml⁻¹ pepstatin A, 1 µg.ml⁻¹ leupeptin.

**Cells**

Cells were obtained by peritoneal lavage of male Sprague-Dawley rats (300g+) and mast cells were then purified to greater than 98% homogeneity by a Percoll step gradient as previously described (Tatham and Gomperts, 1990) and suspended in assay buffer. Approximately 10⁶ mast cells could be prepared from one animal. In preparation for a secretion experiment, the cells were first treated for 5 minutes with metabolic inhibitors (3mM deoxyglucose and 5µM Antimycin A) in order to deplete the cytosol level of ATP.

**Cell Permeabilisation**

Cells were treated with ice-cold SL-O, made up at 1.6 IU.ml⁻¹ in Assay Buffer supplemented with 0.1mM EGTA, for 5 minutes. Under these conditions the SL-O can bind to the cells without causing them to become permeable. The cells were then washed free of unbound SL-O (and impurities) by dilution followed by centrifugation and resuspension in the EGTA-supplemented Assay buffer at 4°C.

In order to initiate permeabilisation and rundown, 20µl of cells were added to 20µl Rundown buffer (as detailed above) and 20µl test protein made up in Replacement or Homogenisation buffer (see above) at 37°C. After the required rundown/permeabilisation time, they were stimulated to secrete by addition of 20µl stimulation (or control) buffer containing Ca.EGTA buffers (3mM) formulated to regulate the concentration of free Ca²⁺ at pCa7 (controls) or pCa5 (estimated using a computer program, Ligandy (Gomperts and Tatham, 1992)) and
GTPγS, at a final concentration of 100μM (or zero) and supplemented with sufficient Mg.ATP to maintain its concentration at 1mM (for details of stimulation conditions refer to figure legends). After allowing a further 20 minute incubation for secretion to occur, the reactions were quenched by addition of 100μl ice-cold Assay buffer (experiments in Chapter 3) supplemented with 10mM EGTA (experiments in Chapters 4 & 5). Cells were then sedimented by centrifugation and the supernatants assayed for released N-acetyl-β-D-glucosaminidase (hexosaminidase) as previously described (Gomperts and Tatham, 1992). Secretion is expressed as the % total cellular content of hexosaminidase, where 100% was determined by cell lysis using 0.2% Triton X-100.

Purification of FOAD II
Deep frozen bovine brains were stored at -80°C. Generally two brains were used for each preparation. These were allowed to defrost for 8 hours at 4°C and then homogenised in Homogenisation buffer (1 litre per 500g) using a Waring blender. The homogenate was centrifuged at 14,400xg overnight at 4°C and the supernatant was then subjected to fractionation by the procedures described below.

The supernatant was initially fractionated by ammonium sulphate precipitation. Material capable of reconstituting the activity of partially rundown cells was found to be present in proteins precipitating at 85% but remaining soluble at 60% of saturation. The cytosol was stirred for 1 hour at 4°C with 60% saturated (NH₄)₂SO₄, then centrifuged at 14,400xg for 30 minutes. The pellet was discarded and the precipitation repeated at 85% saturated (NH₄)₂SO₄. After centrifugation (14,400xg, 30mins at 4°C), the resultant pellet was resuspended in 50ml Homogenisation buffer and applied to an octyl Sepharose column (120ml, XK50 column) equilibrated in 2M (NH₄)₂SO₄, 20mM Pipes pH6.5.

All buffers used for chromatography contained 0.02% NaN₃ to prevent microbial growth and all chromatography steps were carried out on an FPLC at 4°C to reduce proteolysis. Non-adherent material was washed off the octyl Sepharose column with 2M (NH₄)₂SO₄ and this was followed by a linear gradient of (NH₄)₂SO₄ (2-0.5M, at a flow rate of 5ml.min⁻¹), and finally a no-salt wash (20mM Pipes pH6.5) (see Figure 3.2). 10ml fractions were collected. The active
fractions, detected in the run-down assay outlined above, were pooled and
dialysed overnight at 4°C against 20mM Tris-HCl, pH 8.5. This material was then
applied to a Q-sepharose-FF column, (60ml, XK26 column) pre-equilibrated with
20mM Tris-HCl, pH 8.5, and proteins were eluted with a no salt wash followed by
an increasing gradient of NaCl from 0-0.2M NaCl, then a final 1M salt wash. The
flow rate was again 5ml.min⁻¹ and 10ml fractions were collected. The active
fractions were combined and concentrated to 3ml using a YM10 membrane in an
Amicon pressure filtration cell. The samples were exchanged into 20mM Pipes
pH6.9 using NAP-10 columns and then applied to a 5ml HI-Trap SP column. This
was eluted with a no salt wash, then a rising gradient of NaCl from 0-0.4M,
followed by a 1M salt wash, at a flow rate of 2ml.min⁻¹. 1ml fractions were
collected.

At this stage two activities were recovered. The active fractions from the second
peak were pooled and concentrated to 1ml on a Centricon spin filter having a
10kDa cut off. This was injected on to a Superdex G75 preparation grade gel
filtration column (HiLoad 16/60, XK16, volume 122ml) pre-equilibrated in Assay
buffer with no added BSA (flow rate 0.12ml.min⁻¹), and 0.8ml fractions were
collected. The activity eluted as a single peak with an estimated molecular weight
of 43kDa. The active fractions were again pooled and exchanged into 20mM
Tris-HCl, pH 8.5, and then applied to a MonoQ column (1ml) which was eluted
with a no salt wash followed by an increasing gradient of NaCl from 0-0.4M, then
a final 1M salt wash. The flow rate was 0.5ml.min⁻¹ and 0.5ml fractions were
collected. A stimulatory activity and an inhibitory activity were recovered from
this column. The stimulatory activity was then concentrated to 1ml as before using
a Centricon spin filter and re-chromatographed on the G75 column. This was
pre-equilibrated and eluted exactly as outlined for the first G75 chromatographic
step.

Data presented in Figures 3.2-3.6 derives from a single preparation which was
repeated on at least 25 occasions.
Protein sequencing (performed by H. N. M. Freeman)

Protein sequencing samples were pyridylethylated prior to analysis according to a slight modification of a published method (Thomsen and Bayne, 1988). Briefly, the sample was concentrated to dryness on an Amicon 3 (3kDa cut-off) spin filter and resuspended in 70μl guanidine buffer (6M guanidine HCl, 0.25M TrisCl, 2mM EDTA, pH7.5) prior to incubation with 2μl 10% 2-mercaptoethanol in guanidine buffer for 15 minutes under nitrogen in the dark at room temperature. This was followed by the addition of 2μl 4-vinylpyridine in ethanol (1 + 5 vols) and further incubation for 15 minutes under similar conditions. Sample components were separated by reversed phase HPLC (Hewlett Packard 1090) using an Aquapore RP-300 column (10cm x 4.6mm id) and a linear gradient of 0.1% TFA/acetonitrile at 1ml.min⁻¹. Peak fractions were characterised by gel electrophoresis and portions directly sequenced on an ABI 476A protein sequencer or dried and incubated with 50μl of cyanogen bromide solution (7 mg/ml; 70% trifluoroacetic acid) for 18 hours under nitrogen in the dark. The reactions were terminated by addition of 500μl water. The samples were dried and resuspended in tricine sample buffer (Novex) and the protein fragments separated on a 16% tricine gel (Novex) (Schagger and von Jagow, 1987). These were transferred to PVDF (ABI ProBlott) by electroblotting in 10% methanol/10mM CAPS pH 11 (Matsudaira, 1987). Bands were visualised using sulforhodamine B stain and excised for sequencing.

SDS-PAGE analysis

Unless indicated otherwise (e.g. see below), samples for gel analysis were mixed 50:50 with sample buffer (2% SDS, 20% glycerol, 20mM EDTA, 0.01% Bromophenol blue, 120mM Tris-HCl pH 6.8, supplemented with reducing agent, 10% v/v β-mercaptoethanol) and boiled before being separated on 12% SDS-polyacrylamide gels according to the method of Laemmli (Laemmli, 1970). Proteins were visualised either by staining with Coomassie brilliant blue or by silver staining (Morrissey, 1981).
**GTP Overlay of G75 Column Fractions**

Column fractions eluted from the G75 were treated with sample buffer before being separated on a 12% SDS-polyacrylamide gel. Proteins were then transferred to Immobilon membrane (Millipore, Bedford, MA, USA) using a semi-dry blotting system (Pharmacia) run at 15V for 1 hour in blotting buffer (39mM Glycine, 0.0375% SDS, 20% methanol, 48mM Tris). The membrane was blocked by immersion overnight at 4°C in GTP overlay buffer (2μM MgCl₂, 1mM β-mercaptoethanol, 0.3% Tween-20, 1mM Mg.ATP, 50mM Tris, pH7.5), and then for 1 hour at room temperature with agitation. The membrane was labelled by incubation for one hour at room temperature with GTP overlay buffer supplemented with [α³²P] GTP to give a final radioactivity level equivalent to 1μCi/ml. Following this the membrane was washed free of unbound nucleotide using fresh unsupplemented GTP overlay buffer for a total of five washes lasting 10-15 minutes each. After blotting on a paper towel to remove excess buffer, the membrane was wrapped in plastic film and exposed to a phosphorescence imaging plate for 2 hours. GTP binding was analysed using a Fuji phosphorescence imager (Fuji, Japan).

**Preparation of tissue cytosols and cell lysates**

1) **Crude rat brain, bovine brain and rat liver cytosols:**
One rat brain or similar sized samples of bovine brain or rat liver was homogenised using a Dounce homogeniser in 5ml ice-cold Homogenisation Buffer (without ATP or DTT). Homogenates were then treated with approximately 200μM diisopropyl phosphofluoridate for 10 minutes at room temperature, and centrifuged at 8,000xg for 10 minutes at 4°C to separate crude membrane and cytosol fractions. Samples were treated with sample buffer and boiled, separated by SDS-PAGE and analysed by Western blotting (for method see later).

2) **Mast cell, HL60 and Jurkat cell lysates:**
Mast cells from 5 rats, or HL60 or Jurkat cells were resuspended in 150μl of Homogenisation Buffer (without ATP or DTT). The cell suspensions were then treated with approximately 200μM DFP for 10 minutes at room temperature,
taken up in sample buffer and boiled for 15 minutes. Samples were then
vigorously sonicated using a Kontes sonicator and re-boiled in preparation for
SDS-PAGE and analysis by Western blotting.

Immunoblotting

Samples to be analysed were run on 12% polyacrylamide gels and the proteins
transferred to nitrocellulose using either a Pharmacia semi-dry blotting system or a
BioRad wet blotting system (see figure/table legends for details). Membranes were
then probed with either anti-Rac, anti-Rho, anti-G25K, or anti-RhoGDI antibodies
obtained from, and according to the method of Professor Alan Hall (Ridley et al.,
1993), or with commercial antibody preparations of anti-Rac1, anti-Rac2 or
anti-RhoGDI according to the manufacturer's instructions.

Assessment of RhoGDI and Cdc42 leakage from SL-O permeabilised mast
cells

Purified mast cells were treated with approximately 200μM DFP for 10 minutes at
room temperature. They were then washed, treated with metabolic inhibitors and
permeabilised as if in preparation for a normal rundown experiment. Samples of
cells were taken at various times following permeabilisation, immediately
sedimented, supernatants removed, and the proteins present in the supernatant
aggregated by addition of four parts of ice-cold acetone and incubating at -20°C
for approximately two hours. After this, the precipitated proteins were pelleted by
centrifugation, taken up in sample buffer and boiled. These samples were then
separated on a 12% polyacrylamide gel, and the proteins transferred to
nitrocellulose membranes using a BioRad wet blotting system run at 250mA
constant current for 1 hour in Transfer buffer (192mM Glycine, 20% Methanol,
25mM Tris-HCl pH 8.3). Membranes were then probed using either anti-Cdc42Hs
or anti-RhoGDI, both obtained from commercial sources and used in accordance
with the manufacturer's instructions, or anti-Rac obtained from Professor Alan
Hall using the protocol outlined in (Ridley et al., 1993) (NB. Anti-Rac antibodies
were used at a dilution of 1:1000 for this experiment due to general deterioration
of the antibody preparation).
**Purification of recombinant Rac2 (standard method)**

A culture (volume equivalent to 1/10 of the final volume required), inoculated with one colony taken from a streak plate of *E.Coli* containing cDNA encoding GST-Rac2, was grown in Terrific broth supplemented with 50μg.ml⁻¹ ampicillin overnight at 37°C with agitation. This culture was then diluted 1 in 10 in fresh broth (plus ampicillin as before) and grown for 1 hour at 37°C with agitation. Protein production was then induced by the addition of 100μM IPTG, following which the culture was left to grow for a further 4 hours. The bacterial cells were harvested by centrifugation (4,400x g for 10mins at 4°C) and the pellets resuspended in Digestion buffer (50mM NaCl, 1mM DTT, 4mM MgCl₂, 0.02% NaN₃, 20mM Tris-HCl pH 7.4), in a volume equal to 3% of the final culture volume. The cells were disrupted by sonication (MSE Soniprep, with the cells kept cool throughout) and separated into soluble and insoluble fractions by centrifugation (12,000 x g, 10mins at 4°C). The supernatant was added to Glutathione Sepharose beads, pre-washed into Digestion buffer (2ml of a 1:1 suspension of beads in buffer per litre of culture) and incubated at room temperature for 1 hour with agitation. Following this, the beads were sedimented (500 x g, 5mins) and the supernatant discarded. The beads were then washed three times in Digestion buffer to remove unbound material and then once again with Digestion buffer supplemented with 2.5mM CaCl₂. The beads were then resuspended in a volume (equal to five times the bead bed volume) of Digestion buffer supplemented with 2.5mM CaCl₂, 25mM β-octyl glucoside and thrombin (final concentration of 20U.ml⁻¹), and incubated at room temperature for 1.5 hours, following which the beads were pelleted and the supernatant removed and retained. Any cleaved protein remaining associated with the beads was removed by washing (three times) using Digestion buffer, and these washings plus the initial supernatant were pooled. Residual thrombin was removed by addition of 30μl p-aminobenzamidine agarose and incubation at room temperature for 30mins. The supernatant was separated from the beads by centrifugation as before, diluted two-fold in Digestion buffer to dilute the detergent to below its cmc and protease inhibitors (0.1mM PMSF, 1μg.ml⁻¹ pepstatin A, 1μg.ml⁻¹ leupeptin) added. The protein was passed through a 0.2μm filter to remove any remaining beads and dialysed against a total of 2 litres of Digestion buffer (supplemented
with protease inhibitors) for 3-4 hours (with 2 or 3 buffer changes). The protein was then concentrated using an Amicon pressure concentrator (YM10 membrane) to 2ml for application to a G75 preparation grade gel filtration column (Hi Load 16/60 Superdex 75pg) pre-equilibrated in Digestion buffer plus protease inhibitors as before. The column was eluted using a flow rate of 0.5ml.min\(^{-1}\), and 0.8ml fractions collected. Fractions containing rRac2 (eluting between approximately 67-73 ml) were pooled and concentrated using the pressure concentrator as before, and then flash frozen in liquid N\(_2\) and stored at -70°C.

**Mono-Q chromatography of rRac2**

The recombinant Rac2 obtained by thrombin cleavage (following treatment with p-aminobenzamidine beads) was exchanged into 20mM Tris-HCl pH 7.5 plus 1mM DTT and 0.02% NaN\(_3\) using two PD10 buffer exchange columns, and applied to a Mono-Q column (1ml) pre-equilibrated in the same buffer. The column was eluted using a buffer wash followed by an increasing gradient of NaCl from 0-0.4M, and then a final wash of 1M NaCl. The flow rate was 1ml.min\(^{-1}\) and 1ml fractions were collected.

Selected protein-containing fractions were exchanged using NAP-5 columns into Exchange buffer for preactivation and were analysed following preactivation for activity in a fixed rundown time assay using the Ca\(^{2+}\)-only stimulus. The same fractions were also analysed by SDS-PAGE using the standard methods described earlier in this chapter.

**SDS-PAGE analysis of rRac2: alternative treatments**

**Non-reduction:**

Samples were mixed 50:50 with sample buffer in the absence of reducing agent and incubated at room temperature for 30mins. Samples were then loaded and separated by SDS-PAGE using the standard procedure.
Alkylation:

This treatment is understood to protect -SH groups which may otherwise be insufficiently reduced by standard treatments and give rise to partial refolding of some of the protein, resulting in two distinct bands after separation of a pure protein on SDS-PAGE.

Samples were mixed 50:50 with sample buffer (pH 8) supplemented with 26mM DTT, and boiled for 5mins. 10\(\mu\)l iodoacetamide solution (20\% w/v in \(H_2O\)) was then added per 100\(\mu\)l sample and the sample incubated at room temperature for 30mins. Samples were then loaded and separated by SDS-PAGE using the standard procedure.

Urea treatment:

Samples were precipitated with acetone as described above ("Assessment of RhoGDI and Cdc42 leakage from SL-0 permeabilised mast cells"), after which the protein was pelleted by centrifugation and then taken up in Digestion buffer (formulation given under "Purification of rRac2") plus 8M Urea. This solution was incubated at 37\°C for 15mins and then mixed 50:50 with standard sample buffer. The sample was then boiled (or not, refer to figure legend, figure 4.5) for 15mins, following which it was loaded and separated by SDS-PAGE using the standard procedure.

Purification of recombinant T17NRac2-GST

This procedure was identical to that used for purification of wild type rRac2 up until the point of elution of protein from the Glutathione Sepharose, with the following modifications:

- The initial culture was inoculated with a loopful of a concentrated stock of the transformed bacteria.
- The bacteria were not induced. Following dilution, they were simply grown for 5 hours in the absence of IPTG.
- The supernatant obtained following sonication and centrifugation was dialysed against a total of approximately ~2.5l Digestion buffer over a period of 2 hours, with 2-3 buffer changes before incubation with Glutathione Sepharose. This step served to increase the yield of protein by reducing the amount of free
reduced glutathione (GSH) which may occupy binding sites on the beads which
would otherwise be available to bind fusion protein.

Fusion protein was eluted by washing the beads into Glutathione elution buffer
(150mM NaCl, 1mM DTT, 4mM MgCl₂, 0.02% NaN₃, 50mM Tris-HCl pH 8),
and then incubating the beads with an equal volume of 10mM GSH made up in
Glutathione elution buffer, for 10min at room temperature with agitation. This
was repeated three times, and the supernatants pooled. The beads were then
washed three times with elution buffer to remove any remaining fusion protein,
and the washes were added to the elutions. Protease inhibitors (0.1mM PMSF,
1µg.ml⁻¹ pepstatin A, 1µg.ml⁻¹ leupeptin) were added, and the fusion protein was
flash frozen and stored at -70°C.

The percentage of free GST contaminating the preparation of rT17N-Rac2 was
assessed by measuring the relative density of the protein bands detected by silver
staining following SDS-PAGE. This was achieved using a BioRad 1650 Scanning
Densitometer linked to a Shimadzu C-R3A chromatopak monitor.

NB. Attempts to purify non-fused rT17NRac2 by cleavage with thrombin are
detailed in Chapter 4.

Purification of Cdc42/G25K
The procedure was identical to that used for purification of wild type rRac2, with
the following modifications:
• The initial culture was inoculated with a loopful of a concentrated stock of the
transformed bacteria.
• Cultures were grown in LB broth instead of Terrific broth, and grown for 3
hours following induction with IPTG.
• The buffer used up until the thrombin cleavage step was Digestion buffer plus
protease inhibitors (0.1mM PMSF, 1µg.ml⁻¹ pepstatin A, 1µg.ml⁻¹ leupeptin). For
thrombin cleavage, beads were washed into Digestion buffer plus 2.5mM
CaCl₂, and then thrombin was added to a final concentration of 5U.ml⁻¹. This
mixture was incubated for 1 hour at room temperature with agitation.
• Cleaved protein was not dialysed before concentration and gel filtration
Purification of rC-myc-Cdc42Hs and rT17N-Cdc42Hs

These proteins were purified according to a slight modification of the published method (Self and Hall, 1995b), as follows:

A culture (volume equivalent to 1/10 of the final volume required), inoculated with a loopful of a concentrated stock of the transformed E.Coli, was grown in LB broth supplemented with 50μg.ml⁻¹ ampicillin overnight at 37°C with agitation. This culture was then diluted 1:10 in fresh broth (plus ampicillin as before) and grown for 1 hour at 37°C with agitation. Protein production was then induced by the addition of 100μM IPTG, and the culture was allowed to grow for a further 4 hours. The bacterial cells were harvested by centrifugation (4,400 x g for 10mins at 4°C) and the pellets resuspended in Buffer “A” (50mM NaCl, 5mM MgCl₂, 0.02% NaN₃, 50mM Tris-HCl pH 7.5) supplemented with 2mM PMSF in a volume equal to 1% of the final culture volume. The cells were disrupted by sonication using an MSE Soniprep sonicator, and the sonicate centrifuged (12,000 x g, 10mins at 4°C). The supernatant was retained and DTT added to a final concentration of 15mM. Following dialysis against a total of ~3.5l Buffer A (plus 0.1mM DTT) over a period of 2 hours with 2 buffer changes, the supernatant was added to glutathione Sepharose beads, pre-washed into Buffer A (2ml of a 1:1 suspension of beads in buffer per litre of culture). The mixture was incubated at 4°C for 30mins with agitation, the beads were spun down (500 x g, 5mins) and the supernatant discarded. The beads were then washed four times in Buffer A plus 1mM DTT to remove unbound material, and washed into Thrombin Resuspension buffer (150mM NaCl, 5mM MgCl₂, 2.5mM CaCl₂, 1mM DTT, 0.02% NaN₃, 50mM Tris-HCl pH 8). The beads were then resuspended in a volume (equal to 1.5 times the bead bed volume) of Resuspension buffer and thrombin added to give a final concentration of 10U per ml bed volume. After incubation at 4°C overnight with agitation, further thrombin (equivalent to half the previous quantity) was added and the incubation continued for an additional 2 hours. The beads were then pelleted and the supernatant removed and retained. Any cleaved protein remaining associated with the beads was removed by washing (three times) using Buffer A plus 150mM (final) NaCl, and these washings were pooled with the initial supernatant. Residual thrombin was removed by addition of 40μl p-aminobenzamidine agarose per l of original culture and incubation at 4°C for
30mins. The supernatant was separated from the beads by centrifugation as before, and protease inhibitors (0.1mM PMSF, 1μg.ml⁻¹ peptatin A, 1μg.ml⁻¹ leupeptin) added. The protein was passed through a 0.2μM filter to remove any remaining beads and concentrated using an Amicon pressure concentrator (YM10 membrane) to 2ml for application to a G75 preparation grade gel filtration column (Hi Load 16/60 Superdex 75pg) pre-equilibrated in modified buffer A (50mM NaCl, 5mM MgCl₂, 1mM DTT, protease inhibitors as before, 0.02% NaN₃, 20mM Tris-HCl pH 7.5). The column was eluted using a flow rate of 0.5ml.min⁻¹, and 0.8ml fractions collected. Fractions containing rC-mycCdc42 (eluting at ~62-70ml) or rT17N-Cdc42 (eluting at ~66-72ml) were pooled and concentrated using the pressure concentrator as before, then flash frozen in liquid N₂ and stored at -70°C.

**Guanine nucleotide binding assays**

Except for the N17-mutants of Rac2 and Cdc42/G25K, recombinant GTPase concentrations are expressed as their active concentrations, assessed according to their ability to bind [³H]-GTP and [³H]-GDP using a standard filter assay (Self and Hall, 1995b). Briefly, small aliquots of protein (2 or 6 μl) were incubated in a total volume of 800μl Binding assay buffer (50mM NaCl, 5mM MgCl₂, 5mM DTT, 50mM Tris-HCl pH 7.6) with 10mM EDTA and 0.25 nmoles [³H]-GTP (33.4 Ci.mmol⁻¹, 1μCi.ml⁻¹) or [³H]-GDP (38.4 Ci.mmol⁻¹, 1μCi.ml⁻¹) for 10mins at 30°C. Samples were then diluted to 10ml with cold assay buffer (without DTT) and filtered through prewetted 25mm nitrocellulose filters (0.2μm pore size, Whatman, Maidstone, UK) using a Millipore filtration device. The filters were washed with 3ml ice-cold assay buffer (without DTT), for a total of three washes and then dried in air. The radioactivity was determined by scintillation counting. The concentration of active protein binding nucleotide was calculated as follows:

If 1 mol of Rho binds 1 mol of [³H]-GTP, then 1μg Rho should yield 10⁶ dpm (disintegrations per minute) The concentration of the protein sample (mg.ml⁻¹) is calculated using the following equation:

\[
[\text{Protein}] = \frac{\text{cpm} \mu\text{L}^{-1}}{10^6} \times \frac{100}{\text{counting efficiency}}
\]
The binding was measured at two different concentrations of protein (as indicated above), in triplicate, and the experiments were repeated on three occasions (non-tagged Cdc42/G25K, twice).

**Preactivation of GTPases**

Where indicated, GTPases were "pre-activated" by binding GTPγS in Exchange buffer (3mM EDTA, 0.16mM MgCl₂ [free Mg²⁺ ~ 2.75.10⁻⁶M], 1mM DTT, 0.02% NaN₃, 20mM Tris-HCl pH 8) in the presence of GTPγS (1mM), for 10 min at 30°C. Following this, MgCl₂ was added to a final concentration of 4mM (total) and the protein was immediately loaded onto a rapid desalting column (Pharmacia) and eluted with Replacement buffer (assay buffer with no BSA, plus 0.3mM EGTA, 1mM dithiothreitol, 0.1mM phenylmethylsulfonyl fluoride, 1 µg.ml⁻¹ pepstatin A, 1 µg.ml⁻¹ leupeptin). This step served the dual purposes of removing unbound GTPγS and also exchanging the protein into an iso-osmotic buffer (pH6.8) suitable for applying to permeabilised cells.

N17-Rac2 and N17-Cdc42/G25K were used without preactivation after dialysis against Replacement buffer for 15 hours using a BRL Laboratories (Gaithersburg, MD, USA) micro-dialyser (3kDa cut-off).

**Determination of protein concentration**

Protein concentrations quoted in Chapter 3 were determined by the BCA method, using a commercially available kit (Pierce, Chester, UK). Protein concentrations quoted in Chapters 4 and 5 were determined by the method of Bradford (Bradford, 1976). Bovine serum albumin was used as a standard, and sample concentrations were estimated by comparison with a standard curve, constructed using linear regression analysis.

**Statistical analysis**

Where quoted, p values were derived using the t-test, and the data was analysed using the program "Instat" (GraphPAD Software, San Diego, CA, USA).
CHAPTER 3

“PURIFICATION AND CHARACTERISATION OF FOAD II”
INTRODUCTION

The phenomenon of cell "rundown", which occurs as a consequence of prolonged permeabilisation, has already been discussed in some detail (for details refer to Introduction - "The "rundown" assay"). The aim of this study was to identify and purify protein regulators of mast cell secretion using modulation of the rate of run-down as a means of bioassay. Preliminary studies demonstrated that when mast cells are permeabilised and allowed to run-down in the presence of dialysed rat brain cytosol proteins they retain the ability to secrete in response to stimulation by Ca^{2+} and GTPγS for a much longer period than control cells run-down without added protein. From this, it was concluded that brain cytosol would be a promising starting material from which to purify putative regulatory proteins for mast cell secretion. However, in order to obtain a high yield of final product which could then be used in further investigations, it was decided to use bovine brain cytosol as an initial source of proteins.

It should be noted here that the purification work contained in this chapter was undertaken in collaboration with Dr A. J. O'Sullivan. Protein sequencing was carried out by Mr H. N. M. Freeman of the Biomolecular Structure Department, Glaxo-Wellcome Medicines Research Centre, Stevenage, Hertfordshire, UK. All secretion assays carried out on column fractions, and all other work presented in this chapter was carried out solely by myself.
RESULTS

The effect of FOAD II (Factor Of Activation and Degranulation) on mast cell rundown

This chapter describes the purification and characterisation of a factor which is capable of retarding the rate at which permeabilised mast cells lose their ability to secrete in response to stimulation with Ca^{2+} and GTP\gamma S. Figure 3.1 illustrates this loss of activity in a typical “rundown time course” experiment, showing the extent of secretion that can be elicited when cells are stimulated at the time of permeabilisation and that which can be elicited when the cells are stimulated at various times following permeabilisation. At the time of permeabilisation, untreated, or control, cells release typically between 70 and 100% of their contained hexosaminidase. Thereafter, the amount of secretion that can be elicited declines steadily as the time between permeabilisation and stimulation is prolonged. In a typical experiment, as shown in Figure 3.1, the amount of secretion that can be elicited decreases to between 30 and 50% for cells stimulated following 10 minutes permeabilisation, and this declines to zero release when the cells are left permeabilised for approximately 30 minutes before stimulation. It should be pointed out that the actual time course of this “rundown” varies widely between experiments but that the overall pattern of a steady decline in responsiveness with time of permeabilisation remains the same throughout.

The experiment illustrated in Figure 3.1 also reveals the retarding activity that the purified factor has on the rundown of secretory activity. This factor has been named FOAD II (Factor Of Activation of Degranulation). In contrast to the control cells, mast cells permeabilised and incubated in the presence of FOAD II retain the ability to respond to a Ca^{2+} and GTP\gamma S stimulus for a much longer period of time. For example, in the experiment illustrated the protein treated cells were still able to secrete approximately 45% of their hexosaminidase when stimulated 15 minutes after permeabilisation, compared to the meagre 5% elicited from untreated cells stimulated at the same time. Moreover, the FOAD II treated cells remained responsive to stimulation for up to 30 minutes following permeabilisation, only becoming fully refractory to stimulation after 35 minutes. This again contrasts with the untreated cells which retained responsiveness up to
Figure 3.1  Loss of sensitivity (rundown) to stimulation for secretion by mast cells following permeabilisation and its rescue by FOAD II.

Mast cells were permeabilised by treatment with streptolysin-O at 0°C for 5 min, washed and then brought to 37°C in Rundown buffer. A stimulus to secretion (pCa5 with GTPyS, 100μM) was applied at the indicated times and incubation continued for a further 20min, at which time the cells were sedimented by centrifugation and the supernatant assayed for the presence of secreted hexosaminidase. In this experiment the half time for rundown was about 7.5min (open symbols), declining to zero at about 25min. For cells permeabilised in the presence of FOAD II 5μg ml⁻¹ (solid symbols), the initial rate of rundown was similar to the control cells, but from 5min onwards, the rate was retarded so that the half time was about 12.5min, and secretion declined to zero at about 35min. Similar results were obtained on at least three occasions. % Stimulated secretion values indicate stimulated release above control (non-stimulated cells) expressed as a percentage of total cellular hexosaminidase (see Chapter 2 for details). (Error bars represent SEM, n=3, error bars are omitted where too small for inclusion).
15 minutes permeabilisation but then lost responsiveness completely by 20 minutes.

This “rundown rescue” effect was the basis of the assay used in the purification of this activating factor FOAD II. At each stage of the purification process, mast cells were permeabilised and run down for a pre-selected time in the presence of column fractions to test their ability to enhance the extent of secretion in run-down mast cells above that of non-treated cells. In this way the activating fractions could then be selected from contaminating non-active ones to be further purified. In these “fixed rundown time” experiments, the rundown time must be carefully selected. Obviously the cells must be sufficiently run down to expose any retardation of the rundown. Experience has shown that for maximal possible support by FOAD II, mast cells should be left permeabilised before stimulation long enough to allow the corresponding control cells to run down to the point where only a few per cent secretion can be elicited. If the untreated control cells secrete less than 5-10% hexosaminidase following rundown, then it is more likely that FOAD II will have little discernible effect. This is a problem since rundown times vary but luckily this variability is not great on a day to day basis. The rundown rates certainly vary from week to week, and appear to be more rapid during the summer months. In order to select the most appropriate times, a “run-down time course” experiment was always carried out on cells without added protein, similar to that illustrated in Figure 3.1 before proceeding with the purification procedure.

**Purification and identification of FOAD II**

As mentioned in the introduction to this chapter, rat brain cytosol proteins were found to retard the rate of rundown. It had already been shown that this activity could be precipitated by (NH$_4$)$_2$SO$_4$ between 60-85% saturation (A.J.O’Sullivan, unpublished observations). For a practical protein purification procedure it was obviously necessary to transfer to a more copious source such as bovine brain. Unfortunately, dialysed cytosol prepared from frozen-thawed bovine brains exhibited no activity in the rundown assay. However, the proteins precipitating between 60-85% of saturated (NH$_4$)$_2$SO$_4$ again caused retardation of the rundown. This material was taken as the starting point for further purification. An advantage
of using this fraction was that it contained low levels of hexosaminidase which would have interfered in the determination of secretion which was based on measurement of the same enzyme activity.

Proteins precipitated at 60-85% saturation of (NH$_4$)$_2$SO$_4$ were resuspended in Homogenisation buffer, and the level of (NH$_4$)$_2$SO$_4$ made up to 2M by addition of an appropriate volume of 3.4M concentrated stock solution. This was then applied to an octyl Sepharose column which separates proteins according to their hydrophobicity (Figure 3.2). The least hydrophobic proteins i.e. those that do not adsorb to the column matrix were first washed from the column with 2M (NH$_4$)$_2$SO$_4$. Thereafter the adsorbed proteins were eluted from the column, least hydrophobic first, using a declining linear (NH$_4$)$_2$SO$_4$ gradient from 2M down to 0.5M. The column was then washed with buffer containing no salt in an attempt to remove any residual proteins remaining adsorbed to the column. As is seen in the figure, FOAD activity was found in fractions 45-63, eluting at 1.25-0.83M (NH$_4$)$_2$SO$_4$.

These fractions were pooled and dialysed at 4°C overnight against 20mM Tris, pH 8.5, ready for application to a Q-Sepharose Fast Flow (anion exchange) column (Figure 3.3). The column was washed free of unadsorbed proteins and the adsorbed proteins eluted with a linear gradient of NaCl from 0-0.2M, with the residual proteins eluted using a final wash with 1M NaCl. Here, FOAD activity was apparent in the fractions 22-27, eluting at 96-128mM NaCl (the inhibitory activity eluting at above 200mM NaCl was discarded). These fractions were pooled and concentrated to 3ml using a YM10 membrane in an Amicon pressure concentrator, then passed through NAP-10 columns in order to change the buffer to 20mM PIPES pH 6.9. The sample was then loaded onto a HI-Trap SP (cation exchange) column (Figure 3.4), unbound and then bound proteins were eluted using zero NaCl and a linear 0-0.4M NaCl gradient respectively, followed by a 1M NaCl wash to elute any remaining bound proteins. Here it is seen that this procedure separates the stimulatory fraction into two parts. The first, which was called FOAD I, eluting in the first wash with no-salt buffer (fractions 4-9 approximately), does not bind to the column. The second component, FOAD II, elutes later, at 80-220mM NaCl (fractions 17-26). Since the FOAD II emerged with higher specific activity, this component was selected for further purification.
Brain cytosol proteins, precipitated by 60-85% saturated (NH$_4$)$_2$SO$_4$ were resuspended and then made up to 2M (NH$_4$)$_2$SO$_4$ and loaded on to a column of octyl Sepharose (XK50, 100ml). This was eluted with a gradient (2M - 0.5M (NH$_4$)$_2$SO$_4$) and 10ml fractions were collected. Small samples (0.5ml) of the fractions were passed through NAP-5 columns for exchange into Homogenisation Buffer pH6.8, suitable for bioassay, and the rundown time for the activity assay shown here was 15min (error bars were too small for inclusion). Fractions 45-63 (eluting in the range 1.2-0.8M (NH$_4$)$_2$SO$_4$), which contained FOAD activity were pooled and dialyzed overnight against 20mM Tris-HCl, pH 8.5 for loading on to a Q Sepharose (anion exchange) column. Solid symbols indicate protein concentration (A$_{280}$), open symbols indicate FOAD activity (% secretion induced by pCa5 and 100µM GTP$_7$S above that induced in the absence of active protein), continuous line indicates concentration of salt gradient.

Figure 3.2  Chromatography of FOAD on octyl Sepharose.
Figure 3.3 Chromatography of FOAD on Q-Sepharose.

The active material eluted from chromatography on octyl Sepharose was loaded on to a Q-Sepharose column (XK26, 50ml) at pH 8.5 and eluted with an increasing gradient of NaCl (zero-0.2M). 10ml fractions were collected. Samples of the fractions were prepared for bioassay as in figure 3.2, and the rundown time for the activity assay shown here was 15min (error bars were too small for inclusion). Fractions 22-27 (eluting in the range 0.08-0.12M NaCl), which contained FOAD activity were combined and concentrated to 3ml on a YM10 membrane and then exchanged into 20mM Pipes pH6.9 using 3 NAP-10 columns for application to a Hi-Trap SP column. Symbols as for figure 3.2.
Figure 3.4 Chromatography of FOAD on Hi-Trap SP.

The active material eluted by chromatography on Q-Sepharose was loaded on to a 5ml Hi-Trap SP (cation exchange) column at pH6.9 and eluted with an increasing gradient of NaCl (zero-0.4M) and 1ml fractions were collected. Samples of the fractions were prepared for bioassay as in figure 3.2, and the rundown time for the activity assay shown here was 18min (error bars were too small for inclusion). Active material was found to elute in the flow-through buffer (zero salt, non-adherent) and again in fractions 17-26 (0.06-0.24M NaCl). The non-adherent activity was discarded and the active fractions from the second peak were pooled and concentrated to 1ml on a Centricon spin filter (with a 10kD cut off), in preparation for gel filtration chromatography. Symbols as for figure 3.2.
FOAD II fractions 17-26 were concentrated to 1 ml in a Filtron Microsep centrifugal concentrator having a 10 kDa cut off filter and then injected onto a G75 (gel filtration) column, which was eluted in Assay buffer without added BSA (Figure 3.5a). FOAD activity was found to elute out at approximately 58.4 ml, indicating that the molecular weight is approximately 43 kDa\(^1\). The active fractions, and those eluting just before and after (alternate fractions 17-31) were subject to analysis by SDS-PAGE. The active fractions (23 and 25) were found to contain two peptides with molecular weights of 22 and 28 kDa (Figure 3.5b).

Following electrophoretic transfer to an Immobilon membrane they were probed by \([\alpha-\text{^32P]}\) GTP overlay, which showed that the 22 kDa component is a GTP-binding protein (Figure 3.5c).

Attempts to purify FOAD II further, into its two constituent components, abolished its ability to cause retardation of the run-down. This indicated that both peptides are necessary for the support of secretion in the permeabilised cells. If the protein was applied to and then eluted from a Mono-Q (anion exchange) column, however, it could be resolved into a stimulatory and an inhibitory component i.e. one which retards mast cell rundown and one which accelerates it (Figure 3.6a). After washing away unbound proteins as usual with zero salt buffer, the column was eluted with a linear NaCl gradient from 0-0.4M and the stimulatory component eluted at 160-200 mM NaCl whilst the inhibitory component eluted at 220-240 mM NaCl. It was found that the separation of these two components is

\[\text{MW (kDa)} \quad V_e \quad K_{av} \quad (\text{Elution volume}) \quad (\text{see below})\]

<table>
<thead>
<tr>
<th>Standard</th>
<th>MW (kDa)</th>
<th>(V_e)</th>
<th>(K_{av})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ribonuclease A</td>
<td>13.7</td>
<td>79.5</td>
<td>0.460</td>
</tr>
<tr>
<td>Chymotrypsinogen</td>
<td>25</td>
<td>69.6</td>
<td>0.334</td>
</tr>
<tr>
<td>Ovalbumin</td>
<td>43</td>
<td>59.2</td>
<td>0.202</td>
</tr>
<tr>
<td>BSA</td>
<td>67</td>
<td>48</td>
<td>0.060</td>
</tr>
</tbody>
</table>

\(K_{av} = \frac{V_e - V_o}{V_i - V_o}\)

where \(V_i = 122\) ml

\(V_o = 43.3\) ml (determined using blue dextran as a marker)
Figure 3.5  Chromatography of FOAD II by gel filtration on G75

a) The active material eluted by chromatography on HiTrap-SP was loaded (in 1ml) on to a G75 Superdex (gel filtration) column (HiLoad 16/60, preparation grade, XK16, volume 122ml), eluted with Assay buffer (pH6.8, no BSA) and 800μl fractions were collected. Samples from the fractions were assayed for FOAD activity without further adjustment and the rundown time for the activity assay shown here was 18min (error bars were too small for inclusion). The active material found to elute as a 43kDa protein. The active fractions were pooled, concentrated and exchanged using NAP-10 columns into 20mM Tris-HCl pH8.5 for application to a monoQ column. Symbols as for figure 3.2.

For gel analysis and GTP overlay see over....
b) Samples from fractions 17-31 were extracted into standard Laemmli sample buffer under denaturing conditions and the proteins separated on 12% SDS polyacrylamide gels. Visualisation was by silver staining and the gels were photographed using a deep blue filter to enhance contrast.

c) Overlay detection of GTP-binding proteins. Proteins separated on a gel prepared as in (b) (above) were transferred electrophoretically using a semi-dry blotting system (Pharmacia) run at 15V for 1 hour, on to an Immobilon-P membrane. This was probed with [α-32P] GTP as detailed in Chapter 2 and analysed using a Fuji Phosphorescence Imager. The image shows the positions of GTP binding in fractions 22-25.
sensitive to the flow rate of the elution buffer through the column. If the flow rate is raised above 0.5ml/minute, the resolution of the stimulatory peak is lost and the relative proportion of the inhibitory component appears to increase. Analysis of both stimulatory and inhibitory fractions by gel electrophoresis showed the stimulatory factor still to consist of the 22 and 28kDa peptides running together, whilst the inhibitory factor consists of the 28kDa peptide alone (Figure 3.6b). It can be seen that the ratio of 22kDa:28kDa peptides (as marked by silver staining) in the stimulatory fractions is increased over that seen following the G75 step (Figure 3.5b). The stimulatory factor was concentrated to 1 ml using a Filtron centrifugal concentrator, injected onto a G75 column and eluted exactly as before. Analysis of the active fractions by gel electrophoresis now revealed them to consist of the 22 and 28 kDa complex only (Figure 3.6c). From this it appears that the previous Mono-Q column must allow the partial separation of some of the 28kDa peptide away from the complex, and this peptide by itself accelerates mast cell rundown indicating that it is an inhibitor of secretion. The corresponding monomeric 22kDa peptide appears to co-elute from this column with the remaining complex and this can be removed by the subsequent gel filtration step. When applied to the permeabilised cells on its own it appears to be without effect in the rundown assay.

The proteins eluted from the second G75 column were subjected to sequence analysis. From the 22kDa component, four peptides comprising 47% of the total protein were sequenced (Table 3.1). These were found to share 97.8% identity with the human ras-like protein TC25 (human Rac1) and 92.0% identity with human Rac2 (with which Rac1 is 92% homologous) (Didsbury et al., 1989). Unfortunately no sequence could be obtained of the C-terminal (variable) region which would have enabled an unambiguous identification to be made. There are, however, seven specific sequence differences elsewhere which are consistent with Rac1 rather than Rac2, and furthermore no mRNA encoding Rac2 has been found in (human) brain (Didsbury et al., 1989; Reibel et al., 1991)). It is therefore likely that the 22kDa protein is Rac1. Sequence analysis of two peptides of the 28kDa component of FOAD II, representing 28% of the total protein, gave sequences which are 100% identical to those of bovine RhoGDI (Table 3.2).
Figure 3.6  Chromatography of FOAD II on monoQ.

a) The 43kDa protein eluted by gel filtration was loaded on to a 1ml monoQ column. This was eluted with a gradient of NaCl (zero-0.4M) and 0.5ml fractions were collected. Samples were prepared for bioassay as in figure 3.2, and the rundown time for the activity assay was 22min (error bars were too small for inclusion). Two activities, coinciding with the emergence of protein ($A_{280}$ trace) were detected, the first of which (FOAD II) retarded the run-down in the secretion assay, the second being inhibitory. The pooled material from the stimulatory peak was concentrated to 1ml and re-applied to a Superdex G75 column which was eluted as in the legend to Figure 3.5. Symbols as for Figure 3.2.

For gel analysis see over....
b) Samples from fractions 21-28 were extracted into sample buffer under denaturing conditions and the proteins separated on 12% SDS polyacrylamide gels. Visualisation was by silver staining. Note the presence of a protein doublet (22kD and 28kD) in fraction 19 correlating with FOAD II activity, and a single band (28kD) in fraction 22 correlating with the inhibitory activity.

c) SDS polyacrylamide gel analysis of fractions containing FOAD II activity after re-chromatography by gel filtration on Superdex G75. Note the presence of protein doublets (22 and 28kDa) in fractions 23-25. These correlate with FOAD II activity.
**Table 3.1** Sequence analysis and matching of FOAD II peptide with human TC25

Human rac2 (Swiss prot P15153)
Human ras-like protein TC25 (human Rac1) (Swiss prot P15154)

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Rac2</th>
<th>Rac1</th>
</tr>
</thead>
<tbody>
<tr>
<td>MQAIKCVVVG</td>
<td>DGAVGKTCLL</td>
<td>ISYTTNAFP</td>
</tr>
<tr>
<td>QAIKCVVV</td>
<td>DGAVGKTCLL</td>
<td>ISYTTNAFP</td>
</tr>
<tr>
<td>MQAIKCVVVG</td>
<td>DGAVGKTCLL</td>
<td>ISYTTNAFP</td>
</tr>
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<td>QEDYDRLRPL</td>
<td>SYPQTDVFLI</td>
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</tr>
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<td>QEDYDRLRPL</td>
<td>SYPQTDVFLI</td>
</tr>
</tbody>
</table>

Bold type indicates amino acids sequenced, uppercase indicates matches, and lowercase indicates mismatch with published sequence of rac1 obtained from Swiss-Prot Database. * indicates mismatch of sequence with rac2.

**Table 3.2** Sequence analysis and matching of FOAD II peptide with bovine RhoGDI

Bovine RhoGDI (Swiss prot P19803)

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<tr>
<th>Sequence</th>
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<td>SFVLKEGVY</td>
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<td>PMEEAPKGML</td>
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<tr>
<td>PRAEYEFLT</td>
<td>PM-EA</td>
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</tbody>
</table>

Bold type indicates amino acids sequenced, uppercase indicates matches, and lowercase indicates mismatch with published sequence of bovine RhoGDI obtained from Swiss-Prot Database.
Western blotting studies using anti-Rac and anti-RhoGDI antibodies: 
Leakage of RhoGDI from SL-O permeabilised mast cells

In an attempt to determine which form of Rac is present in the purified FOAD II Rac/RhoGDI complex, and also which form of Rac is present in mast cells, Western blotting of the complex and mast cell lysates with Rac1 and Rac2 antibodies was attempted. The commercial antibodies used, which were raised against sequences present in the C-terminal domain of each protein recognised the recombinant forms with good specificity. However, in every one of the five attempts using FOAD II and mast cell lysates, when other tissue and cell samples were also tested, only spurious results were obtained. The results are summarised in Table 3.3. It can be seen that on a number of occasions, even when the antibodies did exhibit reactivity, it was against tissues/cells in which the mRNA for Rac proteins is reportedly absent (Didsbury et al., 1989; Reibel et al., 1991). Secondly, any reactivity that was detected appeared as a band at around 30kDa. Since it had already been shown that the peptide having the primary sequence of Rac migrates at around 22kDa on SDS-polyacrylamide gels (e.g. figure 3.6c), the antibodies did not even appear to be recognising the correct protein. Thirdly, neither antibody recognised the purified Rac in the FOAD II complex on any occasion that it was tested. Also, when the FOAD II complex and extracts of mast cells, rat brain cytosol, and HL60 cells were probed with a commercial anti-RhoGDI antibody in test no. 3, all four, including the purified FOAD II, exhibited reactivity. All four positive results were apparent as bands at approximately 28kDa, exactly as would be expected for this protein. These findings led to the conclusion that the antibodies available to us are not reliable reagents for use in the determination of Rac subtypes. As a result, it is still not proven conclusively whether the FOAD II complex contains Rac1 or Rac2, although the sequence data shown in Table 3.1 and the reports showing that the only form of Rac1 mRNA present in (human) brain codes for Rac1 (Didsbury et al., 1989; Reibel et al., 1991), suggests that the GTPase component of FOAD II is Rac1. In view of the these problems it was decided again to use antibodies offered by Professor Alan Hall (which should react with both forms of Rac) in a further investigation to determine whether Rac is present in mast cells. Alongside this, the extent of any leakage of the protein from permeabilised cells was assessed using the same basic protocol employed in secretion assays, and monitoring the amounts
<table>
<thead>
<tr>
<th>Anti-Rac1</th>
<th>Anti-Rac2</th>
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<td>TEST NO:</td>
<td>N/A</td>
</tr>
<tr>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>FOAD II</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Mast Cells</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
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</tr>
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<td>++</td>
</tr>
<tr>
<td>Jurkat cells</td>
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</tr>
<tr>
<td>Recombinant Rac2</td>
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</tr>
</tbody>
</table>

+ & ++ indicate positive reactions
- indicates no visible reaction
blank indicates not tested

**Table 3.3. Immunoreactivity of commercial anti-Rac1 and anti-Rac2 antibodies with purified and tissue proteins**

Purified and tissue proteins were prepared as indicated in Chapter 2, separated by SDS-polyacrylamide gel electrophoresis (12% gel) and transferred to nitrocellulose membranes. Transfer was achieved using a wet blotting system (BioRad), run at 250mA constant current for 1 hour. Membranes were probed with anti-peptide antibodies against Rac1 and Rac2 supplied by Santa Cruz Biotechnology, Inc., California, USA. Binding was detected using the ECL technique.
of any Rac leaking into the surrounding medium. The extent of any leakage of RhoGDI was also measured. The permeabilised cells were sampled at regular intervals during 20 minutes and the protein present in the external medium precipitated, resolved by gel electrophoresis, transferred to nitrocellulose and probed with antibodies directed against Rac (from Alan Hall) or RhoGDI (commercial sources). Unfortunately it was never possible to detect Rac in the mast cell supernatants. A cluster of non-specific bands was apparent at and above approximately 30kDa, but no bands in the correct molecular weight range were evident (data not shown). Soon after obtaining this result, Professor Hall communicated that the Rac antibody used here had deteriorated upon storage. The blots of the same samples probed with the anti-RhoGDI antibody showed a rapid leakage of this protein from the cells (Figure 3.7). This was more or less complete following 5 minutes of permeabilisation, indicating that RhoGDI is a true cytosolic protein. The presence of RhoGDI in rat peritoneal mast cells also implies the presence in these cells of small GTP-binding proteins of the Rho family. A previous report showed the presence of Rac (using Alan Hall’s antibody before its deterioration), and Rho in mast cells by western blotting, although again the exact form of each protein present was not determined, and the anti-Rac antibody exhibited some cross-reactivity with Rho (Price et al., 1995). (NB. Since these studies were carried out, a new anti-Rac antibody has become available, and this has been used to confirm the presence (but not the subtype) of Rac in mast cells. Rac has also been demonstrated to leak from mast cells within 5 minutes following permeabilisation with SL-O (Brown et al., 1997)).

Characterisation of FOAD II effects on mast cell secretion
Despite not being able to authenticate the presence of Rac, the presence of RhoGDI and the profound effects of the Rac/RhoGDI complex FOAD II on secretion certainly make Rac a strong candidate as a regulator of secretion in mast cells. Figure 3.8 illustrates the concentration-activity relationship of FOAD II in the rescue of secretory run-down. Mast cells were permeabilised as normal and allowed to run-down under non-stimulating conditions for 17 minutes in the presence or absence of increasing concentrations of FOAD II. Cells were then stimulated to secrete with Ca^{2+} (pCa5) and GTPγS (100μM), and the extent of secretion elicited over the following 20 minutes was assessed. In the absence of
Figure 3.7 Leakage of RhoGDI from mast cells permeabilised by treatment with streptolysin-O.

Cells, pretreated with diisopropylphosphofluoridate to prevent proteolysis were treated as detailed in the legend to figure 3.1 and timed samples were removed as indicated. The cells were sedimented and the proteins present in the external salts solution precipitated with acetone at -20°C. They were then separated by SDS-gel electrophoresis on a 12% gel and transferred to nitrocellulose using a wet blot system (BioRad) run at 250mA constant current for 1 hour. The proteins were then probed with an antibody to RhoGDI and immunoreactivity was detected by the ECL technique. The figure illustrates the relative density of each band by densitometry and the inset shows the appearance of the ECL film.
Figure 3.8 Concentration-activity relationship for purified FOAD II (Rac/RhoGDI complex).

Mast cells were permeabilised in Rundown buffer in the presence of various concentrations of FOAD II and allowed to run down for 17 min before being stimulated to secrete by addition of Ca\(^{2+}\) (pCa5) and GTP\(_{\gamma}\)S (100\(\mu\)M) (solid symbols). Similar results were obtained on at least three occasions. Open symbols indicate no-stimulus controls (pCa7, zero GTP\(_{\gamma}\)S). (Error bars indicate SEM, n=3, error bars are omitted where too small for inclusion).
protein the cells released between 15 and 20% hexosaminidase, and this increased up to a maximum of approximately 50% as the concentration of FOAD II was increased in the range 1-5 µg/ml. The optimum concentration is in the range 5-15 µg/ml with an EC50 of 2.6 ± 0.4 µg/ml (50 ± 8nM) (data from four different preparations).

It is already well established that under the conditions used in these experiments, provision of both Ca2+ and an activating guanine nucleotide is essential for mast cell secretion to occur (Howell et al., 1987; Churcher and Gomperts, 1990). Therefore the dependence on these two effectors was tested, allowing the cells to run down (17 minutes) in the presence and absence of FOAD II, and then stimulating with a range of concentrations of Ca2+ and GTPγS (Figure 3.9a). In this experiment very little secretion was elicited in the absence of the FOAD II even when the strongest stimulus (pCa5 plus 100µM GTPγS) was applied. With FOAD II present, some increase in secretion could be elicited at pCa5.25, although only in the presence of the highest concentration of GTPγS (100µM). In the presence of FOAD II, secretion is significantly enhanced at all concentrations of GTPγS when cells are stimulated with the highest concentration of Ca2+ (pCa5). The most striking observation was that the cells exhibit a small (4%) amount of secretion when stimulated at pCa5 in the absence of GTPγS. One possibility is that this is due to GTP, present in the system as a consequence of transphosphorylation between ATP and residual GDP. However, in a separate experiment I was able to show that deoxyUDP, a competitive acceptor in the reaction catalysed by nucleotide diphosphate kinase, was without effect (Figure 3.9b), leading to the conclusion that the small component of secretion induced by Ca2+ alone is not due to the generation of GTP. The FOAD II appears to be able to substitute for GTPγS (to a small extent) in the stimulation of secretion in run-down mast cells.

The effect of FOAD II on the time course of secretion (i.e. the rate of release of hexosaminidase following stimulation) from partially run-down cells was tested. Figure 3.10 shows a typical time course for secretion from cells which have been allowed to run down for 17 minutes before stimulation, either in the presence of absence of FOAD II. Samples of cells were then removed at various times following addition of stimulus and the extent of secretion determined. As shown
Figure 3.9  Effect of FOAD II (Rac/RhoGDI complex) on the requirement for Ca\textsuperscript{2+} and GTP\textgamma{}S for secretion from run-down mast cells.

a) Cells were permeabilised in Rundown buffer and allowed to run down for 17min in the presence and absence of FOAD II (5μg ml\textsuperscript{-1}) before stimulation with various combinations of GTP\textgamma{}S (abscissa) and Ca\textsuperscript{2+}. Solid symbols indicate presence of FOAD II. Points indicate average of 3 separate determinations, error bars were omitted to maintain clarity. Similar results were obtained on at least three occasions. Note the small degree of enhancement of secretion induced by Ca\textsuperscript{2+} (pCa5) alone (zero GTP\textgamma{}S) when the cells are allowed to run down in the presence of FOAD II.

b) dUDP is without effect on the stimulation of secretion by Ca\textsuperscript{2+} alone (pCa5) when the cells are allowed to run down in the presence of FOAD II. Cells were allowed to run down in Rundown buffer in the presence or absence of FOAD II (59μg ml\textsuperscript{-1}) for 13min before being stimulated as indicated, in the presence or absence of dUDP (250μM). (Error bars indicate SEM, n=3).
Figure 3.10 Influence of FOAD II on the time course of secretion from run-down mast cells.

Cells were permeabilised in Rundown buffer and allowed to run down in the presence (solid symbols) or absence (open symbols) of FOAD II (15μg ml⁻¹) for 17min and then stimulated by the addition of Ca²⁺ (pCa5) and GTPγS (100μM). Samples were withdrawn at the times indicated and processed for measurement of secreted hexosaminidase. % Stimulated secretion values indicate stimulated release above control (non-stimulated cells) expressed as a percentage of total cellular hexosaminidase (see Chapter 2 for details). Similar results were obtained on at least three occasions.
in the figure, for untreated mast cells stimulated after a period of rundown, the rate of secretion occurs very slowly, going to completion about 20 minutes after the addition of stimulus. In contrast, secretion from cells stimulated at the time of permeabilisation is generally complete within about 3 minutes (Lillie and Gomperts, 1993). The presence of FOAD II during the rundown serves not only to increase the overall extent of secretion but also to increase the rate at which it occurs. There is an initial lag of approximately 2 minutes during which the rate of secretion is fairly slow (but still faster than that of untreated cells), and then the rate increases rapidly over the next few minutes, gradually slowing, to level off after 20 minutes. In this experiment the maximum amount of secretion was approximately 25%. The enhanced rate of secretion due to the presence of the protein remains much slower than that for cells stimulated immediately at the time of permeabilisation.

Effect of RhoGDI on mast cell rundown
Figure 3.11 illustrates the effect of RhoGDI on the rate of rundown following permeabilisation in mast cells. Mast cells were treated exactly as for the experiment illustrated in figure 3.1, with pure RhoGDI separated by chromatography on Mono-Q as shown in figure 3.6a. When present alone, and in contrast to the effect of the FOAD II complex, this protein accelerates the rate of cell run-down. In this experiment, the cells were capable of secreting only 10% after 15-20 minutes permeabilisation, compared to the 40% from cells incubated without protein for the same period.
Figure 3.11  Loss of sensitivity to stimulation for secretion as a result of permeabilisation (run-down) and its acceleration by RhoGDI.

Cells were treated as detailed in the legend to figure 3.1 and incubated in the presence (solid symbols) or absence (open symbols) of RhoGDI (10µg ml⁻¹) during the run-down period and subsequent incubation. Cells were stimulated by the addition of Ca²⁺ (pCa5) plus GTPγS (100µM) at the times indicated and following a further 20mins incubation were assayed for secreted hexosaminidase as usual. % Stimulated secretion values indicate stimulated release above control (non-stimulated cells) expressed as a percentage of total cellular hexosaminidase (see Chapter 2 for details). Error bars indicate SEM, n=3, error bars are omitted where too small for inclusion. Similar results were obtained on at least three occasions.
DISCUSSION

Upon prolonged permeabilisation, mast cells exhibit a phenomenon called rundown. This is a gradual decline in their responsiveness to stimulation by Ca²⁺ and GTPγS, as shown in Figure 3.1. Rundown has formed the basis of a bioassay employed in the purification of a cytosolic factor from bovine brain cytosol. When tested in this assay, this factor, which has been named FOAD II (factor of activation of degranulation), retards the onset and the rate of mast cell rundown (Figure 3.1). FOAD II was identified by peptide sequencing to be a complex of the small GTP-binding protein Rac1 and RhoGDI (Tables 3.1 & 3.2). Exogenous proteins provided to the permeabilised cells that retard or accelerate the rate of run-down should be considered as candidates, or at least surrogates for the endogenous regulators, capable of insinuating themselves into the pathway to replace others which have been lost by detachment and leakage. For this reason, Rac can be considered as a candidate for GE, the GTPase postulated to mediate effects occurring late in the stimulus-secretion pathway (Gomperts et al., 1986; Gomperts, 1990). As discussed in detail in the introduction (refer to Introduction - “GTP”), secretion from intact mast cells requires the presence of GTP regardless of the nature of the stimulus, and it appears that there are pathways to secretion that are pertussis toxin sensitive (those stimulated by mastoparan or compound 48/80 (Aridor et al., 1990)), and pathways that are pertussis toxin insensitive (those stimulated by Ca²⁺-ionophores or specific antigens (Saito et al., 1987)). The heterotrimer Gα13 was found to be a regulator of the pertussis toxin sensitive pathway (Aridor et al., 1993), and the data presented here must surely make Rac a candidate for GE mediating the pertussis toxin insensitive, GTP dependent pathway to secretion. It remains possible, however, that Rac acts downstream of a pertussis toxin sensitive G-protein.

Although the presence of exogenous Rac has a stimulatory effect on secretion from the rundown mast cell, the experiments presented here do not reveal how it is exerting the effects observed. Exogenous Rac may be acting to replace endogenous Rac which is lost from the cell upon permeabilisation, or it may be able to substitute for another, non-identical but similar factor involved in regulation. Unfortunately, in the early stages of this work the presence of Rac in
mast cells could not be demonstrated, due to the non-availability of suitable antibodies (Table 3.3). While the commercial antibodies against Rac1 and Rac2 recognised the recombinant proteins with good specificity, when tested against tissue extracts the antibodies exhibited multiple and spurious cross-reactions but on no occasion did they recognise any peptides migrating at the correct molecular weight. The most obvious explanation is that the antibodies could only recognise proteins lacking post-translational modification (i.e. the recombinant proteins), especially as the peptides against which the antibodies had been raised were based on the C-terminal sequences. Although the presence and leakage of Rac could not be determined at this stage, (although as mentioned in the results, this has now been achieved, using a new antibody (Brown et al., 1997)), RhoGDI was found to be present in mast cells. This leaks rapidly from permeabilised mast cells, reaching a maximum within 5 minutes (Figure 3.7). Unfortunately, the total cell content of Rho GDI was not measured in this experiment and so the proportion leaking from the cells (and the proportion retained, if any) was not determined.

RhoGDI has been demonstrated to interact with Rho, Rac and G25K (the human homologue of the yeast cell-division-cycle protein Cdc42 (Shinjo et al., 1990)), and is inactive on proteins of other closely related families such as Ras, Rap, and Rab (Ueda et al., 1990; Hiraoka et al., 1992; Leonard et al., 1992). Therefore the presence in mast cells of RhoGDI, coupled to its inhibitory action on mast cell secretion must be regarded as being significant, implying the presence of one or more GTPases of the Rho family which may play a part in the regulation of secretion. Although not known for sure, for the purposes of this discussion, it will be assumed that the effects of the Rac1/RhoGDI (FOAD II) complex are due to these exogenous proteins penetrating the cells and replacing endogenous Rac/RhoGDI (or another related GTPase coupled to RhoGDI) which leaks from the cells upon permeabilisation. This idea is also supported by a report that the constitutively active mutant form of recombinant Rac1 (V12) can stimulate secretion in permeabilised and washed mast cells (Price et al., 1995). Here, significant increases in secretion are reported to occur when secretion is stimulated by Ca^{2+} alone, but these increases occur under conditions (glutamate replacing chloride as the main solution anion) in which GTPγS would be expected to stimulate secretion in the absence of Ca^{2+} when applied to the cells at the time of permeabilisation (Lillie and Gomperts, 1992b). No increase in secretion was
seen to occur in the presence of the recombinant protein when cells were
stimulated with GTPγS alone.

An interesting observation is that while native Rac is without effect on secretion
when provided alone it becomes active when supplied in the form of a complex
with RhoGDI (Figure 3.6). The most likely explanation for this is that the GDI
maintains the Rac in solution and acts as an escort, conveying Rac to the
membrane in response to stimulation, where it undergoes nucleotide exchange and
becomes activated. Rac has been shown to translocate to the membrane upon
activation (Quinn et al., 1993; Abo et al., 1994; el Benna et al., 1994; Heyworth et
al., 1994; Bokoch et al., 1994), while RhoGDI remains in the cytosol, suggesting
that nucleotide exchange and dissociation of the Rac/RhoGDI complex probably
occur prior to the translocation of Rac to the membrane (Quinn et al., 1993; Abo
et al., 1994). However it was later shown that dissociation of the Rac/RhoGDI
complex occurs only in the presence of GTPγS and membranes, and that
membrane binding correlates with exchange of GDP for GTPγS on Rac. A small
fraction of RhoGDI was also found to remain transiently associated with the
membranes following stimulation with GTPγS, which indicates that it does
interact with membranes during Rac translocation, but does not become stably
associated. Since no nucleotide exchange occurs in the absence of membranes it is
likely that there exists a membrane bound exchange factor (Bokoch et al., 1994).
This idea is further supported by the fact that all the experiments on which it is
based were carried out in the presence of high concentrations of Mg^{2+}. Under
these conditions, little nucleotide exchange will occur on Rac in the absence of an
exchange factor (Knaus et al., 1992), yet, as already described, nucleotide
exchange was detected, in response to GTPγS, exclusively in the presence of
membranes (Bokoch et al., 1994). Similarly, in my secretion experiments, the
Mg^{2+} concentrations (1 mM) are such that little nucleotide exchange would be
expected to occur on Rac, but a stimulatory effect is seen in the presence of
Rac/RhoGDI (FOAD II), implying the presence of an exchange factor in these
cells catalysing the activation of Rac. Hence it seems likely that for Rac to exert a
stimulatory effect on secretion in run down mast cells under the conditions used in
my experiments, the protein must be present as a complex with its escort protein,
RhoGDI, to facilitate translocation to the plasma membrane. Once there, it may
interact with an exchange factor to catalyse the exchange of GDP for, in this case, GTPyS in order to become activated. A number of exchange factors for Rac have been identified (Cerione and Zheng, 1996), but of course there are no clues at present as to the identification of the exchange factor which may be mediating the effects seen here.

Membranes are also the site of some of the target systems regulated by the Rho-GTPases, although cytosolic effectors have been identified (reviewed in (Tapon and Hall, 1997)), and so translocation to the plasma membrane may be important for interaction of the activated GTPase with its effector. For example, it is widely accepted that activation of the NADPH oxidase in neutrophils requires translocation of Rac to the plasma membrane and/or membrane cytoskeleton. Once localised, it interacts with its target proteins in the oxidase complex which comprises four proteins including Rac (Bokoch, 1995; Quinn et al., 1993; Abo et al., 1994; el Benna et al., 1994; Sawai et al., 1993) (although it should be pointed out that some contradictory reports state that Rac translocation does not occur or at least is not an absolute requirement for oxidase activity (Philips et al., 1995; Le Cabec et al., 1994)). It seems probable then, that RhoGDI plays an important role in the targeting of Rac to its specific binding site in the membrane where it undergoes nucleotide exchange and/or interaction with specific effector molecules. Studies carried out on the membrane targeting of Rab9 by RabGDI, (which performs a similar function for Rab proteins (Soldati et al., 1993)), indicate that when the Rab protein is solubilised by delipidated serum albumin rather than by RabGDI, indiscriminate membrane association occurs and the protein is less efficient in its activity when stimulated (Dirac-Svejstrup et al., 1994). It is also possible that a dephosphorylation-phosphorylation cycle is involved in promoting the dissociation and re-association of Rho protein/RhoGDI complexes upon activation and inactivation, respectively. RhoGDI purified from bovine neutrophil cytosol in its complex with Rho has been shown to be phosphorylated, and complexes become less stable when treated with a phosphatase (Bourmeyster and Vignais, 1996). Since RhoGDI is likely to be involved in the membrane localisation of Rho proteins, it is interesting to speculate that there may be a membrane bound phosphatase in the vicinity of the postulated Rac-GEF which has a role in the activation of Rho proteins. Upon
activation by GTPγS, RhoGDI/GTPase complexes may undergo dephosphorylation at the membrane which would promote complex dissociation. Although it should be stressed that there is no direct evidence to support this idea, it is supported by the detection of a transient membrane attachment of RhoGDI following Rac activation (Bokoch et al., 1994). Such a step would in turn ensure that Rac is localised for interaction with its GEF. This idea is also supported by the report that RhoGDI inhibits the ability of the human dbl oncogene product to stimulate GDP dissociation from Cdc42Hs (the human homologue of the S. cerevisiae yeast cell-division cycle protein) (Leonard et al., 1992). Hence Cdc42Hs must dissociate from RhoGDI for GEF-catalysed nucleotide exchange to occur, and this may also be the case for other Rho family proteins.

Another possible effect of RhoGDI may be to prolong the activation of Rac. It has been shown that RhoGDI can interact with both the GDP and the GTP-bound forms of Rac (Hancock and Hall, 1993; Chuang et al., 1993; Sasaki et al., 1993), and it appears also that this GDI is capable of weakly, but consistently inhibiting the dissociation of GTPγS from Rac (Chuang et al., 1993) and Cdc42Hs (Leonard et al., 1992). This small but measurable effect may contribute to any stimulatory activities of the Rac/RhoGDI complex upon activation with GTPγS.

The experiments described in this thesis refer to conditions in which the presence of both Ca^{2+} and GTPγS are normally required to stimulate secretion (Lillie and Gomperts, 1992b). However, for partially run-down cells supported by the Rac1/RhoGDI complex (FOAD II) it is possible to elicit a small but significant amount of secretion in the absence of guanine nucleotide (Figure 3.9). This observation raises the possibility that there may exist a state of equilibrium between the soluble, GDI-bound Rho proteins (generally GDP-bound) and the membrane bound Rho proteins (generally GTP-bound), with the equilibrium shifted heavily towards the cytosolic state in resting cells. Upon stimulation of the proteins, the equilibrium would be expected to shift towards the membrane bound state (see over).

Of course, this is at odds with the cycling theory for GTPases which states that all cytosolic GDI-bound Rho protein is bound to GDP and hence inactive, and upon
activation, the GTPases dissociate from GDI and translocate to the membrane, becoming active following nucleotide exchange. However, this does not explain the observation that a small proportion of Rho proteins can be found in the cytosol, plasma membrane, RhoGDI.

membranes of non-stimulated cells (Le Cabec et al., 1994; Heyworth et al., 1994; Ohoka et al., 1991; Polakis et al., 1989; Hall et al., 1993). It also fails to explain the observations that RhoGDI can interact with GTP-bound as well as GDP-bound proteins, as already mentioned. Finally, at the functional level, it fails to explain the (sometimes less efficient) interaction of some Rho effectors with the GDP-bound proteins as well as the GTP-bound forms (Lamarche and Hall, 1994; Tolias et al., 1995; Lancaster et al., 1994; Bokoch et al., 1996). The idea of an equilibrium encompasses these interactions, since an excess of either GDP or GTP-bound protein would be expected to push the equilibrium in the opposite direction, forcing interactions of GDI with the GTP-bound protein, and interactions of effectors with the GDP-bound protein, as has been observed. With respect to my experiments, this is illustrated by the GTPγS independent secretion which occurs from rundown mast cells supplemented with a saturating concentration of Rac1/RhoGDI (FOAD II). (It is unlikely that the complex was purified as a Rac-GTP/RhoGDI complex as the purification process took a total of 3 days, by which time any GTP present on Rac must certainly have been hydrolysed). If an equilibrium does exist, then addition of an excess of cytosolic Rac, in the form of its complex with RhoGDI, must inevitably shift the equilibrium towards the membrane-bound state, forcing some of the GDP-bound
(supposedly “inactive”) Rac into the membrane. Once localised, this GDP-bound Rac may interact weakly with its nucleotide exchange factor and/or effector molecule, resulting in a limited activation of the secretory mechanism, as observed. Of course, for maximal stimulation of secretion in FOAD II treated cells, GTPγS is required along with Ca^{2+} to optimise Rac activity, as is also illustrated in Figure 3.9. The Ca^{2+} may be required to activate the calcium binding protein Ce, postulated to be involved in the activation of secretion in mast cells, possibly by acting as a guanine nucleotide exchange factor (GEF) (Lillie and Gomperts, 1992a; Lillie and Gomperts, 1992b).

FOAD II is capable not only of increasing the amount of secretion that can be elicited from run-down mast cells, but it also increases the rate at which they secrete, as shown in Figures 3.10a and b. For unsupported cells (no FOAD II), the rate of secretion also declines with time following permeabilisation. This is likely to be due, once again, to the leakage of protein regulators of secretion. If this is the case, then the Rac1/RhoGDI complex (FOAD II) may be capable of increasing the rate of secretion by simply replacing the endogenous proteins lost upon permeabilisation, and thereby increasing the efficiency of the secretory mechanism over that of the protein-depleted control cells. The net result is the increased rate of secretion and the concurrent increased extent of secretion, as illustrated in Figure 3.10.

When separated from the FOAD II complex, pure RhoGDI accelerates the rundown of permeabilised mast cells, seen in Figure 3.11. This inhibitory activity, coupled to the demonstration of the presence of RhoGDI in mast cells, is strong evidence that small GTP-binding proteins of the Rho family are involved in the regulation of mast cell secretion. As already discussed, the actions of RhoGDI are directed specifically to GTPases of the Rho family (Ueda et al., 1990; Leonard et al., 1992; Hiraoka et al., 1992). It is able to inhibit the membrane association of Rho proteins, although the efficiency of binding to the GTP-bound form may be no more 10% of that to the GDP-bound form (Sasaki et al., 1993). However, other reports indicate approximate equality in the efficiency of binding to the two forms (Hart et al., 1992; Chuang et al., 1993). The reasons for this discrepancy are unknown, since Sasaki et al. found preferential binding to the GDP-bound form.
even when using the same assay conditions as the groups demonstrating an equal efficiency (Sasaki et al., 1993).

As mentioned earlier, there is also an apparent requirement for phosphorylation of RhoGDI to stabilise GDP-bound Rho protein/RhoGDI complexes, although the state of phosphorylation of GDI complexed to a GTP bound protein has not been investigated (Bourmeyster and Vignais, 1996). However, the efficiency of GDI binding to active Rho proteins may also be linked to its state of phosphorylation, as this appears to play a part in complex formation and dissociation. Bound RhoGDI inhibits nucleotide exchange (Ueda et al., 1990; Hiraoka et al., 1992; Sasaki et al., 1993; Leonard et al., 1992), intrinsic GTP hydrolysis (Chuang et al., 1993; Sasaki et al., 1993; Hart et al., 1992) and hydrolysis catalysed by GTPase activating proteins (GAPs) (Hancock and Hall, 1993; Sasaki et al., 1993; Hart et al., 1992; Chuang et al., 1993). Therefore an excess of free GDI, as in the experiment in Figure 3.11, would be expected to inhibit the activation of GDP bound protein upon addition of GTPγS by binding to it and impeding its interaction with the nucleotide exchange factor, and it would also prevent any activated protein exerting its effect simply by binding to the protein and confining it in the cytosol as a soluble protein/RhoGDI complex. In addition, any protein already localised to the plasma membrane would be solubilised by free RhoGDI to form these cytosolic complexes (Leonard et al., 1992). In effect, the presence of excess RhoGDI will probably serve to shift the equilibrium between cytosolic and membrane bound Rho proteins heavily towards the cytosolic “side” regardless of the nature of their bound nucleotide, thus inhibiting their activity. Like the RhoGDI purified from bovine neutrophil cytosol (Bourmeyster and Vignais, 1996), presumably the purified native RhoGDI used in the experiment illustrated is dephosphorylated, but capable of undergoing phosphorylation by some as yet undefined mechanism to stabilise any RhoGDI/protein complexes formed. This will greatly facilitate the ability of the free protein to bind and stably confine Rho proteins to these soluble complexes.

It is interesting to note that both purified and recombinant RhoGDI is incapable of completely inhibiting mast cell exocytosis in response to Ca^{2+} and GTPγS even at it’s optimum active concentration (Figure 3.11)(Mariot et al., 1996). This implies
that one or more GTP-binding protein(s) that do not belong to the Rho family must also be involved as regulators of this process.

Inhibition by recombinant RhoGDI of secretion from single mast cells in the patch-clamp configuration requires 20 times more protein to produce an effect comparable to that achieved using RhoGDI purified from brain (Mariot et al., 1996). This suggests that a proportion of the recombinant protein may be incorrectly folded, and/or may be unable to undergo the aforementioned phosphorylation and dephosphorylations. This could result in a reduced efficiency of the protein to interact with Rho proteins and maintain them in RhoGDI/Rho protein complexes. In addition, it must be borne in mind that the patch clamped mast cell may differ in some respects from the SL-O permeabilised cells. Patch clamped mast cells do not undergo appreciable rundown in the 20 minutes following patch rupture, presumably because only a single hole is generated in the plasma membrane, in contrast to the many holes covering the entire cell surface which will be generated by SL-O permeabilisation. Also, the intracellular solutions used differ from those used in permeabilised cell secretion assays (for details see (Mariot et al., 1996)), and the patch clamp investigations are carried out at room temperature (20-25°C), whereas the permeabilised cell assays are carried out, generally, at 30 or 37°C. One would expect any rundown to be slowed at the lower temperature. Finally the patch clamped cells are attached to glass coverslips, probably resulting in activation of systems regulating adhesion, on which Rho proteins have been shown to exert regulatory effects (Parsons, 1996), in contrast to cells assayed using permeabilisation, which remain in suspension throughout.

To summarise, the inhibitory activity of an excess of free RhoGDI on mast cell secretion, as shown in Figure 3.11, must be strong evidence of regulation by proteins of the Rho family. I feel that whilst an equilibrium between cytosolic and membrane-bound Rho proteins may exist as outlined, the activities of Rho proteins probably occur according to the cycling theory under "normal" conditions in vivo. However, the regulatory proteins involved are obviously capable of undergoing unexpected interactions (such as interaction of RhoGDI with GTP-bound Rho proteins) in vitro, but this will probably only take place in vivo under very extreme conditions which rarely occur. Obviously it would be very
difficult to determine whether this is the case, but it is certainly true that studies carried out *in vitro* generally employ conditions which can test the absolute limits of protein-protein interactions in order to detect and characterise them. Hence, inhibition by RhoGDI does provide strong diagnostic evidence for a role for one or more members of the Rho family of GTPases as regulators of secretion.

Taking these last points into account, I can now propose a simple mechanism whereby the Rac/RhoGDI complex exerts its effects on mast cell secretion. RhoGDI facilitates activation of Rac in response to the activating signal (Ca^{2+} plus GTP\_yS) by transporting it to membrane binding sites; here it undergoes nucleotide exchange, probably catalysed by a specific membrane-bound nucleotide exchange factor. RhoGDI also encourages inactivation of the protein by binding to and removing the protein from the membrane after GTP hydrolysis, probably GAP-catalysed, has taken place. Unfortunately there are as yet no clues as to the possible downstream effectors/mechanisms by which the activated Rac exerts its effects. In addition, this scheme does not include the reported phosphorylation-dephosphorylation on RhoGDI that facilitates Rac/RhoGDI dissociation and re-association. This is because there are as yet no clues as to how, where and at what stage during the activation/deactivation cycle of the Rho proteins the GDI undergoes these modifications. Further investigation is necessary to reveal the mechanisms underlying these events.
CHAPTER 4

"PURIFICATION OF RECOMBINANT RAC2 AND ITS CHARACTERISATION AS A REGULATOR OF EXOCYTOSIS"
INTRODUCTION

Chapter 3 described the purification of a Rac1/RhoGDI complex, which was shown to be capable of enhancing secretion in rundown mast cells. In order to investigate the effects of Rac on mast cell secretion further, it was necessary to find a way of purifying monomeric Rac (i.e. without its GDI), and it was also necessary to find a more abundant and more amenable source of protein. The logical solution was to use recombinant protein produced in *E.Coli.*, since this should enable easy production and purification of relatively large amounts of Rac.

Although the purified FOAD II complex, isolated from bovine brain, contained Rac1, here Rac2 was used, as this is reported to be the predominant form of Rac in myeloid cells and other cells of haematopoietic origin (Didsbury et al., 1989). Certainly this has been suggested to be the case for neutrophils (Heyworth et al., 1994; Abo et al., 1994). However, the identification of Rac2 in neutrophils was based on immunoblotting using anti-peptide antibodies which identified immunoreactive material migrating with an apparent MW of ~30kDa (Heyworth et al., 1994; Abo et al., 1994). From our sequencing data, we know that Rac1 runs at approximately 22kDa, so these immunodetection data are ambiguous at best. However, in spite of this, the original studies show predominantly Rac2 mRNA in neutrophils (Didsbury et al., 1989) and it has been suggested that the Rac target in the NADPH oxidase of neutrophils interacts preferentially with Rac2 (Dorseuil et al., 1996). It seemed logical, therefore, to use Rac2 in the study of mast cell secretion.

The aim of the work presented in this chapter was to purify recombinant Rac2 (rRac2) and then to use it to explore the role of Rac as a regulator of mast cell secretion. To this end, wild type rRac2 was used, in an attempt to reflect the situation in the intact cell as closely as possible. This could be preactivated or inactivated by prebinding the appropriate guanine nucleotide before application to the permeabilised cells. The dominant negative mutant recombinant T17N-Rac2 (rT17N-Rac2) was also produced and purified, and used as a further test of the proposition that Rac is a regulator of mast cell secretion.
RESULTS

Purification of recombinant Rac2: Initial attempt

Recombinant Rac2 (rRac2) was expressed in and purified from *E. Coli* using a modified version of the glutathione S-transferase (GST) gene fusion vector pGEX-3X (Pharmacia, Uppsala, Sweden). cDNA encoding Rac2 was introduced into the vector and this was then used to transform *E. Coli* strain XL1-Blue MRF' supercompetent cells (Stratagene, La Jolla, CA, USA). Protein expression using the pGEX-3X vector is inducible by IPTG and GST-fusion proteins are expressed, containing an appropriately positioned cleavage site for thrombin. In this way the proteins are easily purified using glutathione Sepharose beads followed by cleavage with thrombin to release the desired protein in its pure form. For the purification of rRac2, a standard method was initially employed for purification of the protein, using a commercially available kit (Pharmacia) designed for the purpose, according to the manufacturer’s instructions. The progress of a typical purification procedure is illustrated in Figure 4.1. SDS-PAGE analysis of samples taken at intervals during the process shows rRac2-GST fusion protein apparent as a band at ~45kDa following disruption of the bacteria, and although some remained in the bacterial pellet (lane CP), it was mainly present in the soluble fraction (lane CS). Following incubation of the GSH beads with the bacterial supernatant, it appeared that some fusion protein failed to bind and this appeared in the supernatant following centrifugation of the bead/bacterial supernatant mixture (lanes pbS1 & pbS2). Analysis of the loosely pelleted beads (lanes pbB1 and pbB2) revealed the fusion protein evident as the gross 45kDa band together with numerous other non-bound proteins which could be removed by washing. rRac2-GST remained bound as shown in lanes pwS1 and pwS2 which contain samples of supernatant after centrifugation following the first bead wash. The majority of the protein had been removed, and the beads were then washed twice more to ensure complete removal; of non-adherent contaminants. Following treatment of the beads with thrombin, the protein released migrated on SDS-PAGE with a MW of approximately 20kDa (Figure 4.1b, lanes ptS1, ptS2, & ptS3), which is lower than would be expected for Rac. Purified Rac1
Figure 4.1 Purification of recombinant Rac2 (rRac2) using the “Bulk GST purification module” (Pharmacia)

*E. Coli* transformed with cDNA encoding Rac2-GST were grown overnight at 37°C with agitation and after dilution, grown for a further 1 hour. Protein production was then induced by addition of 100μM IPTG and the cells grown for a further 4 hours. Cells were harvested by centrifugation, resuspended in PBS and disrupted by sonication after which the lysate was centrifuged to obtain cytosolic and membrane fractions. The supernatant was then incubated with glutathione Sepharose for 1 hour at room temperature after which the beads were washed. rRac2 was purified from the beads by cleavage with thrombin (25 U.Fl−1) for 10min at room temperature after which the free protein was separated from the beads by centrifugation. This cleavage step was repeated three times.

Samples taken at each stage of the purification were treated with sample buffer and separated on 12% SDS-polyacrylamide gels. Protein was visualised by silver staining.

**Key**

This code is used for all the gels presented which illustrate the progress of purification procedures for recombinant proteins (in Chapters 4 & 5). Deviations from this standard code will be stated in the figure legend where appropriate.

In general, upper case indicates sample type, lower case indicates treatment type.

<table>
<thead>
<tr>
<th>Bead</th>
<th>B</th>
<th>Supernatant</th>
<th>S</th>
</tr>
</thead>
<tbody>
<tr>
<td>Membrane</td>
<td>P</td>
<td><em>E. Coli</em></td>
<td>C</td>
</tr>
<tr>
<td>pellet</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>post (i.e. “after”)</td>
<td>p</td>
<td>binding (to glutathione Sepharose beads)</td>
<td>b</td>
</tr>
<tr>
<td>wash</td>
<td>w</td>
<td>thrombin cleavage</td>
<td>t</td>
</tr>
<tr>
<td>glutathione elution removal</td>
<td>g</td>
<td>calcium</td>
<td>Ca</td>
</tr>
<tr>
<td>elution</td>
<td>e</td>
<td>dialysis</td>
<td>d</td>
</tr>
</tbody>
</table>

Numbers given after the sample code indicate duplicate samples obtained and treated identically.
(authenticated by sequence analysis) migrates at approximately 22kDa, as shown in Chapter 3 (Figure 3.6c). On close inspection of the Rac2 amino acid sequence, it was realised that there is a potential cleavage site for thrombin, between arginine and glycine (amino acids 163 and 164, refer to Chapter 3, Table 3.1). The electrophoretic mobility of the protein (20kDa) resulting from the thrombin treatment was consistent with cleavage at this site. Although the same sequence is present in the Rac1 protein, similar problems of purification using the same technique have not been reported.

A further difficulty was due to the use of the PBS buffering system as recommended by Pharmacia. Phosphate is a chelator of Mg\(^{2+}\), and under such conditions of low Mg\(^{2+}\), guanine nucleotides rapidly dissociate from the Rho proteins which causes them to become unstable (Self and Hall, 1995b). Hence an alternative method for purifying rRac2 was sought.

**Standard method for purification of recombinant Rac2**

Finally, I adapted a method (Kwong et al., 1993) applying β-octyl glucoside during the thrombin cleavage step with the aim of altering the conformation of the protein to shield the additional thrombin cleavage site so that cleavage would occur only at the site of fusion with the GST. The gel in Figure 4.2 shows a time course of thrombin cleavage using this method. This method yielded a cleavage product of approximately 24kDa, as might be expected for intact Rac2. Simple examination of the protein bands by eye indicated that the optimum release from the GSH beads occurs after treatment for 1.5 hour with thrombin (lane ptS 1.5). There was still some uncleaved fusion protein (MW approximately 45kDa), and also some free GST (MW approximately 28kDa) and cleaved rRac2 retained on the beads (lane ptB 1.5) and this was released to some extent by washing the beads two or three times with fresh buffer. Close inspection of each lane of supernatant or released protein (ptS) revealed a slight contamination with free GST. It was also evident that the rRac2 was appearing as a doublet (faint band running just behind the main component), rather than as a single discrete band of pure protein. This was possibly due to incomplete reduction of the protein before application to the gel, since Self & Hall reported problems with smearing of recombinant RhoA on polyacrylamide gels which could be overcome by using freshly prepared
Figure 4.2  Time course of thrombin cleavage using the standard rRac2 protocol

Bacterial cells were grown, induced and harvested as in the legend to Figure 4.1. Cells were resuspended in Digestion buffer before sonication, after which the membrane and cytosolic fractions were separated by centrifugation. The supernatant was incubated with glutathione Sepharose for 1 hour at room temperature following which the beads were washed. Protein was purified by thrombin cleavage (20U.ml⁻¹) for the number of hours indicated, in the presence of 2.5mM CaCl₂ and 25mM β-octyl glucoside. Cleaved protein was separated from the beads by centrifugation.

Samples were treated with sample buffer before being separated on 12% polyacrylamide gels. Proteins were visualised by silver staining. Times of thrombin cleavage are indicated above each lane.
sample buffer (Self and Hall, 1995b). However, subsequent preparations continued to yield a doublet, even when fresh β-mercaptoethanol was used in fresh sample buffer.

**Effect of recombinant Rac2 on mast cell secretion: Preliminary investigations**

In spite of these problems of purification, the recombinant protein was used in preliminary secretion experiments to find out if there might be an effect of the rRac2 similar to that of FOAD II. When permeabilised mast cells were incubated with recombinant, but only partially purified Rac2, there was no discernible effect on the amount of secretion elicited by Ca$^{2+}$ and GTPγS at any time during the rundown of permeabilised mast cells (not shown). However, when the protein was preactivated by binding GTPγS before presentation to the permeabilised cells, it retarded the rate of rundown of the response to Ca$^{2+}$ and GTPγS (Figure 4.3). More interesting than this, however, the cells were seen to secrete in response to a Ca$^{2+}$-only stimulus (although the extent of secretion was much reduced compared to that elicited by the dual stimulus). Normally, (as already discussed in Chapter 3), under the conditions of these experiments permeabilised mast cells have an absolute requirement for the presence of GTPγS to stimulate secretion (unless of course FOAD II is present - refer to Figure 3.9a). The finding of Ca$^{2+}$-induced, GTPγS-independent secretion was therefore very interesting and provided an additional set of circumstances by which the activity of rRac2 preparations could be tested. However, in the knowledge that the rRac2 preparation remained impure I was for the moment reluctant to place too much reliance on these activities.

**Attempts to resolve the heterogeneous rRac2 preparation using a chromatographic approach**

The most obvious explanation for the distinct double band appearance of rRac2 on SDS gels was that each band represented a different form of the rRac2 protein, one obviously slightly larger than the other, the difference being approximately 1-2kDa. In an attempt to separate these two forms, the protein from one preparation was subjected to gel filtration on a G75 column. This also served to a) remove the contaminating free GST, b) remove the contaminating detergent, and c) ensure complete removal of residual thrombin remaining after
Mast cells were permeabilised by treatment with streptolysin-O at 0°C for 5 min, washed and then brought to 37°C in Rundown buffer (pH6.8) containing Mg.ATP (1mM) and sufficient EGTA (0.1mM) to suppress Ca^{2+} to below pCa8. At various times (indicated) samples were removed and stimulated by transfer to solutions containing Ca.EGTA (3mM) to regulate pCa5 (or pCa7 for non-stimulated control cells) in the presence or absence of GTPyS (100µM). The incubation was continued for a further 20min, at which time the cells were sedimented by centrifugation and the supernatant assayed for the presence of secreted hexosaminidase. Cells were stimulated (or not) by addition of: for non-stimulated cells, Ca^{2+} buffer (pCa7, △), for stimulated cells either the Ca^{2+}-only (pCa5, ○,●), or the dual stimulus (pCa5 plus GTPyS, 100µM, ○,■). Open symbols indicate absence, solid symbols indicate presence of rRac2 preactivated by binding GTPyS before application to the cells (8 µg.ml^{-1}, total protein concentration assessed by Bradford protein assay). Similar results were obtained on at least three occasions. (Error bars indicate SEM, n=3, error bars are omitted where too small for inclusion).
Figure 4.4  Chromatography of rRac2 by gel filtration on G75

Following thrombin removal using p-aminobenzamidine agarose, rRac2 from a standard preparation was dialysed against 4 x 11 Digestion buffer over ~4 hours before being concentrated to 2ml and applied to a G75 Superdex (gel filtration) column (HiLoad 16/60, preparation grade, XK16, volume 122ml), pre-equilibrated in Digestion buffer plus protease inhibitors as detailed in Chapter 2. The column was eluted using this buffer and 800μl fractions collected.
p-aminobenzamidine treatment. The resulting elution profile, shown in Figure 4.4, shows that the rRac2 elutes at 70.4ml as a single symmetrical peak consistent with a pure protein of apparent MW of 22.9kDa (for G75 calibration data see Chapter 3 “Results”). In spite of this, it still separated into two components when analysed by SDS-PAGE (shown later, Figure 4.5, lane pG75TA). There were two other distinct peaks. The first, eluting just after the void volume at 44ml, probably consisted of multimeric protein present in the injected sample. The second, eluting at 59.6ml (39.5kDa), probably consisted of free GST, although the apparent molecular weight was not consistent with this since the molecular weight of GST is 26kDa (however, it was later confirmed that GST does migrate on gel filtration at this apparent molecular weight - see Figure 4.14c). Analysis of the pooled rRac2-containing fractions revealed no contamination, unlike the cleaved protein before gel filtration (Figure 4.2, ptS lanes), and so this gel filtration step procedure was integrated into the purification procedure.

It was possible that the proteins comprising the doublet of rRac2 differed by only a handful of amino acids, insufficient to resolve on the G75 column. This might be expected if one band was a clipped form of the intact Rac peptide, resulting from protease or endopeptidase activity. By contrast, a difference of only a few amino acids between the two bands of Rac might be expected to result in overall charge differences sufficient to allow their separation by ion exchange chromatography. A portion of the recombinant protein (doublet) from gel filtration was subject to chromatography on a MonoQ anion exchange column (not shown) and when tested in the fixed run-down time assay (Ca^{2+}-only stimulus), stimulatory activity was confined to a single fraction containing rRac2, as indicated by SDS-PAGE analysis. However, the protein still separated into a doublet (approximately 24kDa) as before (not shown).

**Treatment of the heterogeneous rRac2 preparation for SDS-PAGE using a number of methods**

As both gel filtration and strong ion exchange had failed to separate the two bands of protein, I considered the possibility that while the protein was indeed homogeneous, its migration on the SDS gel was anomalous. One explanation for a
pure protein migrating on a polyacrylamide gel as a pair of close running bands could be because the protein is only partially reduced when treated with sample buffer for SDS-PAGE analysis. If this was the case, then part of the protein would remain partially folded and migrate at a different rate to the fully reduced protein. As already described, the use of fresh sample buffer and/or fresh β-mercaptoethanol in the sample buffer had been unsuccessful in eradicating this problem. Therefore alternative methods of protein treatment for analysis by gel electrophoresis were investigated.

Figure 4.5 shows a gel on which samples of purified rRac2 were analysed following different treatments which were carried out prior to sample loading. A fresh batch of rRac2 was purified and the appearance of the protein released from the GSH beads is shown in lanes 3, 4 and 5. Following thrombin removal with p-aminobenzamidine, portions of the protein were removed and treated as detailed in the Figure legend (lanes TA-TD inclusive). None of these samples migrated as a single band, not even the non-reduced sample in lane ptTB. The samples in lanes ptTC and ptTD, the alkylated and boiled urea treated samples, respectively, appear to have migrated more slowly than the others but the doublet persists. There appears to be an additional band of protein in the alkylated sample, migrating at approximately 16kDa. This may have resulted from proteolysis during treatment. There was no change in the migration pattern when the protein was dialysed in preparation for gel filtration (lane pd). Nor did any of these treatments have any effect in resolving the doublet when applied following gel filtration (lanes pG75 TA-pG75 TD2). Again, the alkylated and boiled urea-treated samples (lanes pG75 TC and pG75 TD, respectively) have migrated more slowly than the rest, and there is a 16kDa fragment in the alkylated sample. The sample in lane pG75 TD2 is a post-G75, urea-treated sample which was not boiled, but the gel shows that this precaution (intended to reduce the formation of cyanate ions) had no effect.

The conclusion to be drawn is that, contrary to initial expectations, the appearance of the protein on polyacrylamide gels, is an accurate representation of the peptide composition of the purified rRac2. The two components of the rRac2 doublet must
Figure 4.5 SDS-PAGE analysis of rRac2 samples following various treatments

Samples of rRac2 from a standard preparation carried out as detailed in Chapter 2 (either following thrombin removal or following gel filtration on G75, as indicated) were treated as follows before being separated on a 12% polyacrylamide gel (for details of treatments see Chapter 2):

- **TA** Treatment A: Reduced in sample buffer as normal
- **TB** “ B: Non-reduced
- **TC** “ C: Alkylated
- **TD** “ D: Urea treated
- **TD2** “ D2: Urea treated, not boiled

(NB. All other samples shown were treated with reducing sample buffer as normal).
Proteins were visualised by silver staining.
differ slightly from each other, but not sufficiently to allow separation by either gel filtration or ion exchange chromatography.

**Selection of bacterial cells for homogeneous rRac2 protein production**

Clearly both forms of rRac2 were being produced by the *E. Coli* as GST-fusion proteins. Up until this point, when culturing *E. Coli* for recombinant protein production, fresh medium had been inoculated with a loop of transformed bacteria, which had been stored frozen in 15% glycerol at -70°C. Although this method is widely used, ideally a sample of the concentrated bacterial stock should be grown up on agar, to allow a single colony to be picked off to inoculate the medium. This ensures that the resulting culture originates from a single cell and it avoids the (rare) possibility of growing up different forms of the proteins arising from random mutations. Although there is a risk of selecting a colony arising from a mutant bacterium, the likelihood of this occurring must be remote.

In order to eliminate the possibility that the heterogeneous rRac2 preparations were occurring as a result of the production of a mutant in addition to the wild type protein, streak plates were prepared of the stock culture and a number of mini-cultures set up using individual colonies. The purified proteins resulting from each of these cultures were analysed by SDS-PAGE as shown in figure 4.6a. Lane ptrS a shows the protein purified from a culture innoculated directly from the concentrated stock as before. As expected, the protein has migrated as a doublet (although on this occasion the upper band is more prominent). Lanes ptrS b - ptrS h show the proteins generated from each of the 7 mini-cultures B-H and it can be seen that of these, C and D ((lanes ptrS c & ptrS d) generated protein that migrated as a single band. Lanes ptrS b (culture B), and ptrS e - ptrS h (cultures E-H) all contain protein migrating as a doublet, which indicates that a mutation is not responsible for the two forms of protein purified. If some bacterial cells were producing one form of protein and others a different form, then one would expect to have seen only a single band in these lanes. Possible reasons for the persistence of the doublet in these samples are given in the discussion to this chapter.

The protein products of cultures A-D were tested for activity in the rundown assay following preactivation with GTPγS (Figure 4.6b). All of them enhanced secretion elicited by Ca²⁺ plus GTPγS above that of control cells incubated without protein.
Figure 4.6 Analysis of rRac2 proteins purified from individual *E. Coli* cultures grown from selected single colonies

Bacteria were streaked onto an agar plate and grown overnight at 37°C. Single colonies from this plate were used to grow cultures (overnight at 37°C) which were then grown, induced, and the resulting rRac2 purified using the standard protocol as detailed in Chapter 2, but omitting the final stages of dialysis followed by gel filtration on G75.

NB. Sample A was grown from a loop of mixed stock culture as a control, and the protein purified and treated exactly as for each of the other cultures.

a) SDS-PAGE analysis. A sample of the protein purified from each culture (designated a-h) was treated with sample buffer and separated on a 12% polyacrylamide gel. Protein was visualised by silver staining.

For secretion assay see over....
b) The effect of each protein on mast cell secretion. Mast cells were permeabilised as detailed in the legend to Figure 4.3 and allowed to run down for 15min in the presence or absence of preactivated rRac2 protein purified from each of the cultures A-D. Cells were then stimulated by the addition of either the Ca^{2+}-only (pCa5, dark grey bars), or the dual stimulus (pCa5 plus 100μM GTPγS, light grey bars). Control cells received calcium buffer to regulate pCa7 (black bars). Relative protein concentrations were very roughly estimated using the A_{280} trace obtained during desalting following preactivation, concentrations of proteins from cultures B-D inclusive were found to be approximately equal whilst the concentration of the protein from culture A and found to be approximately 1.75 times that of the other cultures. (Error bars indicate SEM, n=3).
Whatever the difference between the two forms of Rac comprising the doublet, it evidently has no effect on the ability of the protein to enhance secretion in rundown mast cells. The frozen stock of culture "C" was retained and the others discarded. For all subsequent purifications of rRac2, initial cultures were propagated by innoculation with single colonies taken from a streak plate of bacteria from culture "C", in an attempt to ensure the resulting protein preparations remained homogeneous. Figure 4.7a shows the progress of the revised rRac2 purification following the binding of rRac2-GST to GSH beads (samples up to this point always appeared as shown in Figure 4.1). Samples of these beads following treatment with thrombin are analysed in lanes ptB1 and ptB2. There is a prominent band appearing at approximately 28kDa, almost certainly the residual GST on the beads following release of the rRac2. Other bands are apparent, migrating at molecular weights below 28kDa. The band immediately below the GST has migrated at an apparent molecular weight of approximately 24kDa and this is likely to be cleaved rRac2 remaining associated with the beads. Multiple bands of protein are also apparent, at molecular weights below both GST and rRac2 which probably correspond to proteolytic products of both proteins. It is also possible that some rRac2 is still cleaved at its internal cleavage site during the thrombin treatment despite the presence of the octyl β-glucoside. However, these products remain associated with the GSH beads, since they are not apparent in lanes ptS1 and ptS2, which show the protein released during the thrombin treatment. There is a prominent single band, at approximately 24kDa corresponding to the rRac2 band in the post-thrombin beads sample. There is also a faint band at approximately 36kDa, probably thrombin. Lanes ptrS1 and ptrS2 contain the same samples (as ptS1 and ptS2, respectively) following p-aminobenzamidine agarose treatment to remove thrombin. The 36kDa band is reduced, although there is still a faint band alongside the purified rRac2. It was found that addition of protease inhibitors was necessary immediately following the p-aminobenzamidine agarose treatment to inhibit the action of residual thrombin. If these were omitted the purified rRac2 appeared as a smeared band indicative of proteolysis (illustrated in Figure 4.8b). With addition of protease inhibitors, the protein appeared as a single discrete band at the correct molecular weight (Figure 4.7a, lane pd) though it should be noted that the elution profile obtained from gel filtration of the homogeneous rRac2 preparation appeared exactly as for the heterogeneous preparations, with rRac2 eluting at
Figure 4.7  Purification of homogeneous rRac2 using the standard protocol

Bacteria from culture “C” (see Figure 4.6a) were streaked onto an agar plate and grown overnight at 37°C. A single colony from this plate was then used to grow a culture from which rRac2 was purified using the standard protocol as in the legend to Figure 4.2. Samples were taken at stages of the purification and treated with sample buffer before being separated on 12% polyacrylamide gels. Proteins were visualised by silver staining.

a) Samples ptB1 to pG75 (samples taken before this point appeared identical to those in Figure 4.1).

b) Appearance of rRac2 which has not been treated with protease inhibitors following purification. Note the smearing of the band.
70.4ml (MW~22.9kDa) (not shown). The homogeneity of the preparation following gel filtration was once more confirmed by SDS-PAGE (Figure 4.7a, lane pG75). The proportion of active rRac2 capable of binding nucleotide was measured using a filter binding assay (Self and Hall, 1995b). In agreement with previous reports, approximately 1/10 of the total protein, measured using the Bradford assay, was capable of binding \[^{3}H\]-GTP or \[^{3}H\]-GDP (either nucleotide gave very similar results).

**Effect of recombinant Rac2 on mast cell secretion**

The effect of the purified recombinant Rac2 on mast cell secretion was investigated. As isolated, it had little effect on the rate of rundown in permeabilised mast cells when cells were stimulated by the dual stimulus (pCa5 plus 100µM GTP\(\gamma\)S) and no secretion could be elicited with the Ca\(^{2+}\)-only stimulus (figure 4.8). These findings confirmed the results of the preliminary experiments discussed earlier. In contrast (as shown in figure 4.9), when preactivated by binding GTP\(\gamma\)S the rRac2 (active protein 1.5µg ml\(^{-1}\), 70nM) enhanced secretion in response to the dual stimulus and reduced the rate of rundown at times beyond 10mins following permeabilisation such that it was still possible to induce 15% hexosaminidase release even 40 minutes later.

More striking still was the observation that for cells provided with preactivated rRac2, it became possible to induce exocytosis by provision of Ca\(^{2+}\) alone (see Figure 4.9). In the experiment illustrated, this caused the release of 40% of total hexosaminidase when the Ca\(^{2+}\) stimulus (pCa5) was provided at the time of permeabilisation, and this then declined so that no secretion could be elicited when the stimulus was provided at times beyond 30min.

Concentration-effect relationships for the GTP\(\gamma\)S loaded rRac2 in the permeabilised mast cells were established using Ca\(^{2+}\) alone (figure 4.10a) and also Ca\(^{2+}\) plus GTP\(\gamma\)S (figure 4.10b) as stimuli. Protein concentrations are given as the "active" concentrations (as determined using nucleotide binding assays). For cells stimulated by Ca\(^{2+}\) alone, in this experiment, the enhancing effect of preactivated rRac2 became apparent at about 0.03 µg ml\(^{-1}\) (1.4nM) and optimised at about 0.6µg ml\(^{-1}\) (28nM) supporting an extra 60% of Ca\(^{2+}\)-induced secretion over that
Figure 4.8 Time course of rundown of permeabilised mast cells in the presence and absence of rRac2

Cells were permeabilised as detailed in the legend to Figure 4.3, in the presence and absence of rRac2 (14.8μg ml⁻¹, total protein concentration). A stimulus to secretion was applied at the indicated times and incubation continued for a further 20min, at which time the cells were sedimented by centrifugation and the supernatant assayed for the presence of secreted hexosaminidase. ○, ● Cells stimulated with Ca²⁺ (pCa5) and GTPγS (100μM); □, ■ Ca²⁺ alone (pCa5); solid symbols indicate presence of rRac2. Similar results were obtained on three separate occasions. % Stimulated secretion values indicate stimulated release above control (non-stimulated cells) expressed as a percentage of total cellular hexosaminidase (see Chapter 2 for details). Error bars indicate SEM, n=3, error bars are omitted where too small for inclusion.
Figure 4.9  Time course of rundown of permeabilised mast cells: Effect of homogeneous rRac2, preactivated by binding GTPγS

Cells were permeabilised as in the legend to Figure 4.3 and allowed to run down in the presence and absence of preactivated rRac2 for the indicated times before stimulation by transfer to solutions containing Ca.EGTA (3mM) to regulate pCa5 in the presence or absence of GTPγS (100μM). In this experiment the concentration of active rRac2 was 1.5μg ml⁻¹. ○,● Cells stimulated with Ca²⁺ (pCa5) and GTPγS (100μM). ▲,△ cells stimulated with Ca²⁺ (pCa5) alone; solid symbols indicate presence of preactivated rRac2. % Stimulated secretion values indicate stimulated release above control (non-stimulated cells) expressed as a percentage of total cellular hexosaminidase (see Chapter 2 for details). (Error bars indicate SEM, n=3, error bars are omitted where too small for inclusion). Similar results were obtained on four separate occasions.
Figure 4.10 Concentration-effect relationships for the enhancement of secretion by preactivated rRac2 stimulated by a) Ca\(^{2+}\) alone and b) Ca\(^{2+}\) plus GTP\(_7\)S

ai) Purified mast cells were permeabilised as detailed in the legend to Figure 4.3 and allowed to run down for 7 minutes before applying the Ca\(^{2+}\) (pCa5) stimulus or Ca\(^{2+}\) buffer to regulate pCa7 (control). Protein concentrations, expressed as active protein binding \([^{3}H]\)-GTP, were applied according to a \(\times 2\) serial dilution scheme. Points indicate % stimulated secretion above control. Results are expressed as means ± SEM. \((n=4)\), error bars are omitted where too small for inclusion. Similar results were obtained on four separate occasions. ■, stimulated cells; □, unstimulated control cells.

aii) Data presented as a Hill plot. For secretion stimulated by Ca\(^{2+}\) alone, slope \(p=1.8\) \((r=0.99, n=3\text{ data points})\). Similar results were obtained on four separate occasions.

bi) Purified mast cells were permeabilised as detailed in the legend to Figure 4.3 and allowed to run down for 17 minutes before applying the Ca\(^{2+}\) plus GTP\(_7\)S stimulus or Ca\(^{2+}\) buffer to regulate pCa7 (control). Results are expressed as means ± SEM. \((n=4)\), error bars are omitted where too small for inclusion. Similar results were obtained on four separate occasions. ■, stimulated cells; □, unstimulated control cells.

bii) Data presented as a Hill plot. For secretion stimulated by Ca\(^{2+}\) plus GTP\(_7\)S, slope \(p=1.6\) \((r=0.99, n=5\text{ data points})\). Similar results were obtained on four separate occasions.
elicited from the untreated cells. The active concentration range, \((EC_{50} \sim 0.33 \mu g\ m l^{-1},\) or \(15nM,\) as drawn) encompasses approximately one decade. Analysis of the data from the experiment illustrated in Figure 4.10a according to the logistic expression

\[
\% \text{ secretion} = \frac{100 \times [\text{Rac}]^p}{([\text{Rac}]^p + K^p)}
\]

gave a value of \(p = 1.8\) (least squares fitting for values of secretion lying between 5\% and 95\% gives \(r = 0.99,\) \(n = 3\) data points). In two other experiments that were analysed, values of \(p = 1.9\) and 2.3 were derived and in one further experiment the slope of the Hill plot was so steep that there were no data points within the useful range (5-95\% secretion) for analysis.

When the dual stimulus (Ca\(^{2+}\) plus GTP\(\gamma\)S) was applied (see Figure 4.10b), the range of concentrations of preactivated rRac2 enhancing secretion in the run-down cells may have been slightly extended, but from the data obtained, this cannot be stated conclusively. For the experiment illustrated, \(p = 1.6\) (\(r=0.99,\) \(n=5\) data points, Figure 4.10c). However, in spite of the much longer period of time allowed for the run-down to occur, the mid-point concentration for preactivated rRac2 (\(EC_{50} \sim 0.23 \mu g\ m l^{-1},\) or \(11nM,\) as drawn) was not significantly shifted with respect to stimulation by Ca\(^{2+}\) alone.

The experiment illustrated in Figure 4.11 was carried out in an attempt to determine whether the rRac2 mediates its effects on mast cell secretion via the effector mediating the similar effects which occur in response to FOAD II (the Rac1/RhoGDI complex isolated from bovine brain cytosol). Rac1 and Rac2 are 92\% homologous (Didsbury et al., 1989), and probably interact with common effector proteins especially as they are identical in their effector binding regions (refer to Chapter 3, Table 3.1, residues 32-40 by analogy to Ras (Sigal et al., 1986)). However, there is some dispute about this, especially since it has been suggested that effectors can interact with Rac via other regions as well as the effector region (Freeman et al., 1996; Diekmann et al., 1995). Unfortunately, there is little work comparing the effector interactions of these two proteins, though it has been reported that the interaction of Rac with the p67phox protein in the neutrophil NADPH oxidase, Rac2 shows a higher affinity for the protein than Rac1. This fits neatly with the finding that Rac2 comprises >95\% of the total Rac protein.
Figure 4.11  Enhancement of secretion due to saturating concentrations of FOAD II in the presence and absence of an optimal concentration of preactivated rRac2

a) Purified mast cells were permeabilised as detailed in the legend to Figure 4.3 and allowed to run down for 7 min in the presence or absence of FOAD II (36 μg.ml⁻¹) +/- preactivated rRac2 (1.45 μg.ml⁻¹ active protein), and then stimulated by addition of the Ca²⁺-only stimulus (pCa5, grey bars). Non-stimulated control cells received Ca²⁺ buffer to regulate pCa7 (black bars). Results are expressed as means +/- SEM (n=4). Similar results were obtained on 5 separate occasions.

b) Purified mast cells were permeabilised as detailed in the legend to Figure 4.3 and allowed to run down for 17 min as in a) above and then stimulated by addition of the dual stimulus, Ca²⁺ (pCa5) plus GTPγS (100 μM) (grey bars). All other details are as in a) above.
in neutrophils (Abo et al., 1994), although these studies were carried out using anti-peptide antibodies which recognised a peptide migrating at ~30kDa, which is therefore unlikely to be Rac. However, since it has been inferred that Rac2 is the most abundant form of Rac in myeloid cells (Didsbury et al., 1989), it is quite possible that Rac2 interacts more strongly with mast cell effector molecules than does Rac1 in the regulation of secretion. In addition, it has been shown that Rac1 (again, no studies using Rac2) can interact with some effector proteins regulated by the related Rho family protein Cdc42, such as the PAK like kinases (reviewed in (Tapon and Hall, 1997)). Although the possibility of secretory regulation by Cdc42 had not been raised at this stage, this was obviously worthy of consideration, especially as Rho (Price et al., 1995) had already been suggested to be capable of enhancing the secretory response of mast cells. It was therefore possible that Rac1 (as part of the FOAD II complex) exerts its effects on mast cell secretion through a Cdc42 effector pathway rather than through a Rac-specific effector, especially if, as already mentioned, it is true that in mast cells as in neutrophils, Rac1 has a decreased affinity (compared with that of Rac2) for an endogenous Rac effector. Testing the effects of the two proteins together would hopefully provide a way of determining whether they act on common or distinct effectors.

The two forms of Rac (recombinant Rac2 and purified Rac1 as FOAD II, its complex with RhoGDI) were tested for activity both separately and together in a fixed rundown time assay. For cells incubated with FOAD II, there was a little secretion in response to the Ca\(^{2+}\)-only stimulus, as before (see Chapter 3, Figure 3.9a) while the preactivated rRac2 supported 31% secretion, and when both activated rRac2 and FOAD II were provided together during the rundown, this increased to 58%. Obviously, one or both of the proteins was potentiating the activity of the other. Figure 4.11b shows the effects of the same treatments on mast cells stimulated with the dual stimulus, Ca\(^{2+}\) and GTP\(\gamma\)S. In this experiment, FOAD II allowed an increase in the secretory response over the control level (30%) to 68% while cells run down in the presence of preactivated rRac2 alone released 48%. Most interesting however, was the finding that cells incubated with both proteins during the rundown showed a slightly enhanced response (74% release in this experiment) over that elicited from cells incubated with either protein on its own. Although small, it is statistically significant (p<0.01, n=5
separate experiments), and may indicate that each protein can interact with a
different downstream effector. This possibility will be considered further in the
discussion.

**Purification of recombinant T17N-Rac2: Optimising growth conditions**

The next stage of this investigation involved the production and purification of the
dominant negative mutant rT17N-Rac2 from *E.Coli*, in order to test its effects (if
any) on secretion from permeabilised mast cells. Preliminary attempts at purifying
this protein using the method employed for the wild type rRac2 purification
yielded no pure protein. SDS-PAGE analysis of samples taken throughout this
purification revealed that the majority of the fusion protein remained in the
insoluble membrane fraction following bacterial cell disruption and centrifugation
(Figure 4.12b, lanes P16 and S16). Attempts to solubilise the protein using Triton
X-100 (1%) were unsuccessful. In an attempt to overcome this problem, a series
of mini-cultures were grown under different conditions in which temperature,
level of induction, and total time of growth following induction were varied. I
decided on this approach because experience indicates that the lower the level of
foreign protein present in the bacterial cells, the less likely it is to be repackaged in
inclusion bodies, rendering it insoluble. This experiment was carried out in order
to optimise the conditions of induction and growth in order to obtain the
maximum amount of soluble protein. Figure 4.12 shows an SDS-PAGE analysis
of the soluble and insoluble fractions of disrupted bacterial cells grown under
various conditions. At first glance, these all appear more or less identical.
Lanes containing samples of insoluble membrane fractions (P1-P16) exhibit
heavier, more prominent staining than their corresponding soluble cytosolic
fractions, analysed in the lanes immediately to the right of the corresponding
membrane fractions. The gels in Figures 4.12a show samples from bacteria which
were grown at 30°C, those in Figures 4.12b show those grown at 37°C. Those in
Figures 4.12a (top) and 4.12b (top) were grown for 3 hours following induction,
whilst those in Figures 4.12a (below) and 4.12b (below) were grown for 4 hours.
Lastly, in each gel, there are samples from four separate cultures, each grown with
a different level of induction, from left to right: 0, 10, 50, and 100μM IPTG.
Figure 4.12 SDS-PAGE analysis of bacterial membrane and cytosolic fractions from T17NRac2-producing E. Coli grown under various different conditions

E. Coli transformed with cDNA encoding T17NRac2-GST were grown overnight at 37°C with agitation and after dilution, grown for a further 1 hour. Following this, cells were grown and induced for a number of hours following induction with a concentration of IPTG as indicated below. Cells were then harvested and membrane (P) and cytosolic (S) fractions separated as in Figure 4.2. Samples of each were then treated with sample buffer and separated on 12% polyacrylamide gels. Proteins were visualised by Coomassie brilliant blue staining:

a) Cells grown at 30°C for 3 hours (samples 1-4) or 4 hours (samples 5-8) after induction with 0 (samples 1 & 5), 10 (samples 2 & 6), 50 (samples 3 & 7), or 100 (samples 4 & 8) μM IPTG.

For SDS-PAGE analysis of samples from cells grown at 37°C see over....
b) Cells grown at 37°C for 3 hours (samples 9-12) or 4 hours (samples 13-16) after induction with 0 (samples 9 & 13), 10 (samples 10 & 14), 50 (samples 11 & 15), or 100 (samples 12 & 16) μM IPTG.
The rT17N-Rac2-GST fusion protein is apparent as a prominent band migrating at approximately 45kDa. In agreement with the results obtained before, when the cells were grown for 4 hours at 37°C following induction with 100µM IPTG, the resulting fusion protein remained entirely within the insoluble membrane fraction. The fusion protein was present in all the membrane samples analysed, though to a lesser extent in the samples grown at 30°C for 3 hours and with levels of induction <100µM. In contrast, there appeared to be little or no fusion protein in any of the supernatant samples, except for the one from bacteria grown without induction for 4 hours at 37°C (Figure 4.12b, lane S13). This indicated that a basal level of rT17N-Rac2 expression can occur in the absence of chemical induction. The maintenance of low protein levels appeared to ensure that at least some of the protein remained in the cytosol. From here on, the bacteria were grown under these conditions, without the inducer, to ensure the presence of soluble protein for purification.

Use of the standard wild type rRac2 protocol for purification of recombinant T17N-Rac2

Now that the conditions for bacterial production of soluble rT17N-Rac2 had been optimised, purification was once again attempted using the standard protocol for wild type rRac2, as before, with the slight difference that the supernatant obtained from the disrupted cells was dialysed before incubation with the glutathione-Sepharose beads. This step was incorporated in order to reduce the amount of free GSH which may be present in the sample and which will occupy binding sites on the beads, therefore reducing the efficiency of fusion protein-bead binding. Following this, the purification was carried out exactly as for wild type rRac2 but it became evident at the gel filtration stage that no protein had been eluted (not shown). Figure 4.13 shows the SDS-PAGE analysis of samples taken throughout the purification process. Fusion protein was present (as usual, migrating at approximately 45kDa) in the soluble bacterial fraction (Figure 4.13a, lane CS), none was lost during dialysis (4.13a, lane pd), and following incubation of this supernatant with glutathione-Sepharose beads most was attached (4.13a, lane pbB) with only a little fusion protein remaining in the supernatant (4.13a, lane pbS). The fusion protein remained firmly attached when the beads were washed (Figure 4.13b, lane pwS). Lane ptS of the same gel contains a sample of
Figure 4.13  SDS-PAGE analysis of samples taken during an attempt to purify recombinant T17N-Rac2 (rT17N-Rac2) using the standard rRac2 method

Bacteria were grown overnight at 37°C with agitation and after dilution, grown for a further 5 hours without induction. Following this, cells were harvested and then treated as in the legend to Figure 4.2, with the exception that the bacterial supernatant was dialysed before incubation with glutathione Sepharose. Samples were taken at each stage and treated with sample buffer before being separated on 12% polyacrylamide gels. Proteins were visualised by silver staining.
supernatant following thrombin treatment of the beads, and lane ptwS a sample of the material washed from the beads in the first wash following this treatment. No protein was released although a sample of the beads taken following this first wash (lane ptwB) shows that cleavage of at least some of the fusion protein (~45kDa) had occurred since prominent bands at approximately 28kDa and 24kDa were now apparent. These probably represent free GST and rT17N-Rac2, respectively. Other bands, presumably proteolytic peptide fragments, were apparent below 21kDa. Repeated washing failed to remove any protein from the beads (Figure 4.13b, lane ptwS) to which the cleaved protein remained adhered (right hand adjacent lane, ptwB).

**Elution of recombinant T17N-Rac2-GST fusion protein followed by thrombin cleavage and GST removal by glutathione Sepharose**

During the next purification attempt, the complete fusion protein was eluted from the beads with reduced glutathione, and this was then cleaved in solution with thrombin. The resulting mixture was then run through a column of Glutathione-Sepharose to remove the free GST. Two attempts were made using this procedure. During the first, the protein precipitated occurred during the treatment with thrombin, and this was thought to be due to the lack of DTT and MgCl₂ in the buffer used for the glutathione elution and thrombin cleavage steps. As already remarked, Rho proteins rapidly lose bound nucleotide under these conditions and become unstable (Self and Hall, 1995b; Self and Hall, 1995a). They also appear more stable in a reduced environment.

For the second attempt, both DTT and MgCl₂ were included in the buffer used for the glutathione and thrombin treatments. The progress of purification was once again analysed using SDS-PAGE which revealed that as before, soluble fusion protein was present which became bound to the beads but was not be removed by washing. Since the samples taken up to this point appeared identical to their equivalents in Figure 4.13, these are not shown, but samples taken from this point onwards are shown in Figure 4.14a. Lanes pgS1 e1 and pgS2 e2 contain samples of supernatant following the first elution of protein from the beads with reduced glutathione, lanes pgS1 e3 and pgS2 e3 contain supernatant samples following the third elution. The most prominent protein migrated at approximately 45kDa, and
Figure 4.14 Elution of rT17N-Rac2-GST fusion protein followed by thrombin cleavage and removal of GST using glutathione Sepharose

Bacteria were grown, harvested and bound to glutathione Sepharose beads as in the legend to Figure 4.13. Following binding, rT17N-Rac2-GST was eluted using reduced glutathione (10mM), and this protein was cleaved in solution with thrombin (20U.ml⁻¹) in the presence of CaCl₂ (2.5mM) and β-octylglucoside (25mM). Following thrombin removal and dialysis, the protein was passed through a glutathione Sepharose column (e1 - flow through) which was eluted with sequential applications of Digestion buffer supplemented as follows:

- e2 - unsupplemented buffer
- e3 - buffer + 1M NaCl
- e4 - unsupplemented buffer
- e5 - buffer + 20% ethylene glycol
- e6 - buffer + 40% ethylene glycol

Eluates 1 & 2 were pooled, concentrated to 2ml and applied to a Superdex G75 gel filtration column (HiLoad 16/60, preparation grade, XK16, volume 122ml) which was eluted as in the legend to Figure 4.4.

a) Samples taken throughout this procedure were treated with sample buffer before being separated on 12% polyacrylamide gels. Proteins were visualised by silver staining.

For second gel and A₂₈₀ elution profile from gel filtration chromatography see over....
b) Samples taken throughout this procedure were treated with sample buffer before being separated on 12% polyacrylamide gels. Proteins were visualised by silver staining. “PPT” indicates protein which precipitated during the procedure.

c) A\textsubscript{280} elution profile from gel filtration chromatography. Protein was seen to elute at 44ml and 60ml and the protein constituting the latter peak was found to be GST (see (b) above)
this is likely to be the eluted rT17N-Rac2-GST fusion protein. Other bands present below this are probably products of proteolysis, but one, migrating at around 28kDa appears very prominent. This is likely to be free GST. Lanes pgB1 and pgB2 of the same gel contain bead samples taken following the glutathione elutions. The composition of the proteins remaining is similar, if not identical to that which has been eluted off the beads, as would be expected. Washing the beads after elution removes some eluted protein remaining transiently associated with the beads, which is analysed in lanes pgwS1 and pgwS2. During the thrombin treatment, a precipitate again started to form, implying that the presence of DTT and MgCl₂ was an ineffective precaution against this occurrence. In order to minimise any ionic interactions which may have been causing the precipitation, extra NaCl was added (final concentration, 150mM) and this did seem to halt the precipitation. Following thrombin treatment of the eluted protein in solution, and removal of the precipitate, three proteins were detected by SDS-PAGE, shown in Figure 4.14b, lane ptS. These migrated at approximately 45, 28, and 24kDa, and presumably corresponded to rT17N-Rac2-GST, cleaved GST, and cleaved rT17N-Rac2, respectively. The sample in lane ptrS is the same sample following p-aminobenzamidine treatment, and little change had occurred. The precipitated protein was also analysed, shown in Figure 4.14b, lane PPT. It appears that the proteins comprising this precipitate were the same as those appearing following thrombin cleavage, as would be expected. Note that although it appears that the larger part of the protein was precipitated, this is not really the case, since the protein in the precipitate sample was obviously highly concentrated compared to that remaining in solution. The soluble protein was then dialysed (Figure 4.14b, lane pd: the sample is diluted but its composition is unaltered) before being passed through a column of glutathione Sepharose, from which the eluate was collected. The column was then washed through with 3 column volumes each of (in order of application) unsupplemented buffer, buffer plus 1M NaCl, unsupplemented buffer, buffer plus 20% ethylene glycol, buffer plus 40% ethylene glycol, and finally unsupplemented buffer again. The eluates were collected with each wash and samples of each were analysed by SDS-PAGE, shown in Figure 4.14b, lanes el-e7 inclusive. The only eluate in which protein was apparent was eluate 1, which was the flow-through eluted when the protein solution was initially applied to the column. Three bands of protein were present, corresponding to the three bands of protein present in the solution applied to the column (lane pd). The amount of free
GST present seemed to have declined considerably, as did the amounts of fusion protein and rT17N-Rac2. Eluates 1 and 2 (eluate 2 was included since it was merely a buffer wash, and may have contained a small amount of protein) were pooled, dialysed and then concentrated for application to the G75 gel filtration column. This column was eluted as normal, and the $A_{280}$ profile is shown in Figure 4.14c. There were two peaks of protein, one eluting at approximately 44ml, probably multimeric protein which normally elutes at this point during gel filtration of purified recombinant wild type Rac2 (compare Figure 4.4). The other, more prominent peak eluted at approximately 60ml, giving the protein an approximate MW of 40kDa (for G75 calibration data see Chapter 3 “Results”). The fractions comprising this peak were pooled and analysed by SDS-PAGE, shown in Figure 4.14b, lane pG75. This protein was seen to consist entirely of free GST, leading to the conclusion that this protein moves anomalously during gel filtration. The small amount of rT17N-Rac2 present in elutions 1 and 2 from the glutathione Sepharose column appeared to have been lost at some point.

At this stage it seemed likely that some of the rT17N-Rac2 had been lost during its passage through the glutathione-Sepharose column, especially as there was already evidence that the purified protein can associate fairly tightly with these beads. Figure 4.14b illustrates that this association was even stronger than first thought since the various eluting buffers applied to the column failed to elute any additional protein. As a result the amount of rT17N-Rac2 loaded onto the G75 may have been so small that it failed to register on the trace. It seemed logical to conclude that glutathione Sepharose is wholly unsuitable for the purification of rT17N-Rac2 and another method of removing GST following thrombin cleavage was sought.

**Elution of recombinant T17N-Rac2-GST fusion protein followed by thrombin cleavage and removal of GST by gel filtration**

For the next purification attempt, the fusion protein was again eluted with glutathione and cleaved with thrombin in solution. These procedures were carried out exactly as in the previous attempt. Note that this time, in order to prevent precipitation, the concentration of NaCl during the glutathione elutions was maintained at 150mM and the extent of precipitation appeared much reduced.
Figure 4.15 Elution of rT17N-Rac2-GST fusion protein followed by thrombin cleavage and gel filtration

Bacteria were grown, harvested, and the rT17N-Rac2-GST fusion protein eluted and then cleaved with thrombin in solution as in the legend to Figure 4.14, with the NaCl concentration in the elution buffer increased to 150mM. Following thrombin removal, this protein was dialysed, concentrated to 2ml, and then applied to a Superdex G75 gel filtration column (HiLoad 16/60, preparation grade, XK16, volume 122ml) which was eluted as in the legend to Figure 4.4. Protein was seen to elute at 44 & 60ml and this latter peak was found to contain pure GST.

Samples taken throughout this procedure were treated with sample buffer before being separated on 12% polyacrylamide gels. Proteins were visualised by silver staining.
SDS-PAGE analysis of samples taken during the first stages of the purification appeared exactly as before (see Figure 4.14a and 4.14b), and are not shown here. Figure 4.15 shows the SDS-PAGE analysis of samples taken throughout the remainder of the purification. Lane pgS shows the successful elution of fusion protein from the beads, and some free GST is also apparent. Lane pgB shows the beads following the elution, again with some fusion protein and GST left attached. Lane pt shows the protein solution following thrombin treatment. Cleavage has produced free GST and free rT17N-Rac2 as expected, although some intact fusion protein remains as usual. Lane ptrB shows the beads following glutathione elution, including the protein which precipitated during the elution and was spun down with the beads. The fusion protein and both cleaved constituents were present in this sample. The protein sample following p-aminobenzamidine treatment is shown in lane ptrS, with all three proteins still present. In an attempt to remove the contaminating proteins from the pure rT17N-Rac2, the protein solution was concentrated in an Amicon pressure concentrator and applied directly to the G75 column, with no further processing. In this way it was hoped that the rT17N-Rac2 could be successfully isolated since it had had no further contact with Glutathione Sepharose. The appearance of the A$_{280}$ trace (not shown) was almost identical to the trace obtained in the previous purification (Figure 4.14c); there was the usual peak of multimeric protein eluting at 44ml, and a main peak eluting at 60ml (MW~40kDa) consistent with the protein being pure GST yet again. This was confirmed by SDS-PAGE, in Figure 4.15, lane pG75 (the other bands present below the GST are presumably proteolysis products). From this it appeared that rT17N-Rac2 was also adhering to the gel filtration matrix, and, as with the glutathione Sepharose, could not be removed by simple washing. However, it was hoped that it could be displaced by washing with buffered NaCl (2M, one column volume) and then with 0.1M NaOH (one column volume). It was not anticipated that any rT17N-Rac2 eluted by these procedures would be suitable for use in activity assays but the exercise was nevertheless carried out in an attempt to confirm the suspicion that the protein was adhering non-specifically to the gel filtration medium. The A$_{280}$ was monitored continuously during this process, and although changes in absorbance were detected, no peaks characteristic of eluting proteins appeared at any stage. Samples taken at the points where changes of absorbance occurred were analysed by SDS-PAGE but no protein was apparent (not shown). The most obvious conclusion must be that the rT17N-Rac2 was
sticking to the gel filtration column and was thereafter irremovable even in the face of the extreme conditions applied. In addition it was concluded that the precipitation which still occurred during glutathione elutions was probably a response to the higher pH (8.0) which is normally used. For this reason I decided to attempt the glutathione elution at pH 7.4, as normally used in the purification of rRac2, and I also decided not to cleave the protein, but to leave it as the GST-fusion protein since the rT17N-Rac2 is so difficult to handle in its free form. The free GST which was inadvertently purified in this last purification was retained (after concentration and freezing) to use as a control in any subsequent experiments carried out using the rT17N-Rac2-GST fusion protein.

Elution of recombinant T17N-Rac2-GST fusion protein followed by gel filtration

Figure 4.16a shows the SDS-PAGE analysis of samples taken from the glutathione elution stage onwards in this next purification (again, all samples up to this stage appeared exactly as before and are not shown). The glutathione elution was carried out at pH 7.4 i.e. glutathione was made up in digestion buffer with the NaCl concentration increased to 150mM. The supernatants taken after the first glutathione elution were analysed in lanes pgS1 and pgS2. No protein had been eluted. All three elutions were carried out as usual, and the beads washed afterwards as usual. Lanes pgwS1 and pgwS2 contain samples of these washings, there was a little fusion protein present in both, and a little free GST in one (pgwS1). Lanes pgwB1 and pgwB2 show the beads following completion of both elutions and washes. A large amount of fusion protein had been retained, along with some free GST. Lane pg contains a sample of the pooled elutions and washes and there appears to be no protein despite some being present in the first wash (lanes pgwS1 and pgwS2). The sample was concentrated in a Filtron centrifugal concentrator and a crude test for protein carried out on the concentrated sample indicated that little or no protein was present. In the assumption that the failure to elute protein was due to the altered pH, the beads were washed into pH 8.0 buffer and the glutathione elution repeated. Samples from this set of elutions were analysed (Figure 4.16b). Lanes pgS1 and pgS2 contain samples of supernatant following the first elution and both fusion protein and free GST were present (in low quantities) confirming that the elution is sensitive to pH. Following three
Figure 4.16 Elution of rT17N-Rac2-GST fusion protein followed by gel filtration

a) Bacteria were grown, harvested, and the rT17N-Rac2-GST fusion protein eluted as in the legend to Figure 4.15 but using glutathione (10mM) buffered at pH 7.4. Little protein was found to elute, so the elution was repeated using pH 8.0, shown in:

b) Elution of fusion protein using glutathione (10mM) buffered at pH 8.0. The eluted fusion protein was concentrated to 2ml and applied to a Superdex G75 gel filtration column (HiLoad 16/60, preparation grade, XK16, volume 122ml) which was eluted as in the legend to Figure 4.4.

Samples taken throughout this procedure were treated with sample buffer before being separated on 12% polyacrylamide gels. Proteins were visualised by silver staining.
elutions as usual, the beads still retained considerable fusion protein plus free GST (lanes pgB1 and pgB2), which could be removed by further washing (lanes pgwS1 and pgwS2). However, judging by the amount of fusion protein remaining on the beads compared to that remaining after the first elution, little fusion protein was released in total, (Lanes pgwB1 and pgwB2, and pgB1 and pgB2, respectively). Lane pg shows a sample of the final solution before concentration for gel filtration. Both fusion protein and free GST were detected even at this dilution although in lesser amounts than would be expected had the elution been successful first time around (i.e. had pH 8.0 had been used initially). The protein was concentrated and injected onto the gel filtration column and the resulting trace (not shown) showed no evidence of any protein. The obvious conclusion to draw was that the protein is still very "sticky", even when coupled to GST, and had probably adhered to a surface, possibly the membrane used in the concentrator, and/or the surfaces of plasticware (tubes, etc.) used during the procedure.

Crude purification of recombinant T17N-Rac2-GST

In the face of all these difficulties it was decided to elute the fusion protein using glutathione exactly as before, and then to use it directly with no attempt at further purification or concentration. At least this would ensure that the minimum amount of protein would be lost by adherance which evidently increases with every step of the procedure. The final sample of protein obtained from glutathione elution and bead washings, analysed by SDS-PAGE, is shown in Figure 4.17. The elution, carried out at pH 8.0 once again, released both the fusion protein and free GST, as before. Since this preparation was to be used in secretion assays with no further processing, it was important to establish the extent of contamination of the fusion protein with free GST. This would then allow the appropriate control experiments to be carried out with the free GST which had been (fortuitously) purified previously (Figure 4.15a). Scanning densitometry of the bands in the SDS-PAGE gel (Figure 4.17) indicated that the free GST comprised about 30% of the total protein (Figure 4.18). Attempts to assess the extent of nucleotide (GDP) binding as had been accomplished for the wild type rRac2 (Self and Hall, 1995b) were
**Figure 4.17  Crude preparation of rT17N-Rac2-GST**

Bacteria were grown, harvested, and the rT17N-Rac2-GST fusion protein eluted as in the legend to Figure 4.15. A sample of this was treated with sample buffer before being separated on a 12% polyacrylamide gel. Proteins were visualised by silver staining. This fusion protein was flash frozen in liquid N\textsubscript{2} and stored at -70°C for use in secretion experiments.
Figure 4.18  Scanning densitometry of SDS-PAGE separated rT17N-Rac2-GST and GST

The bands visualised in Figure 4.17 were subject to scanning densitometry using a BioRad 1650 Scanning Densitometer linked to a Shimadzu C-R3A chromatopak monitor. The resulting trace is shown above, and it shows that the free GST (migrating at ~28kDa) comprised approximately 30% of the total protein.
unsuccessful. Very little binding was detectable (three attempts, each with six separate determinations). The reason for this is unknown, and unfortunately, due to time limitations, could not be investigated further.

**Effect of recombinant T17N-Rac2 on mast cell secretion**

The rT17N-Rac2-GST and free GST were tested for activity in the rundown assay (Figure 4.19). In these experiments, the cells were allowed to run down briefly (7 minutes), sufficient to allow the protein to penetrate the cells while ensuring a high level of release so that any inhibition by the proteins would be more apparent. As can be seen, secretion stimulated by the dual stimulus (Ca\(^{2+}\) plus GTP\(\gamma\)S) can be inhibited by rT17N-Rac2-GST as its concentration is elevated above 1\(\mu\)g/ml. Since I was unable to apply the standard measurements of guanine nucleotide binding to the rT17N-Rac2, the concentrations recorded in Figure 4.19a represent totals (determined using the Bradford assay), not active material. The highest protein concentration applied was 160\(\mu\)g/ml, at which concentration the level of secretion was inhibited by \(\sim 45\%\). GST, applied as a control, was without effect up to 66\(\mu\)g/ml, shown in Figure 4.19b, confirming the specificity of the inhibition due to the rT17N-Rac2.
Figure 4.19  Inhibition of GTPγS stimulated secretion by rT17N-Rac2-GST.

Purified mast cells were permeabilised as detailed in the legend to Figure 4.3 and allowed to run down for 7 minutes before stimulation by Ca²⁺ (pCa5) and GTPγS (100µM).

a) Effect of rT17N-Rac2-GST. Note that the rT17N-Rac2 preparation is contaminated with about 33% GST. Concentrations given are total protein concentrations.

b) Effect of GST applied over a similar range of concentrations.

Results are expressed as means ± SEM (n=4), error bars are omitted where too small for inclusion. Solid symbols indicate cells stimulated with Ca²⁺ (pCa5) and GTPγS (100µM); open symbols indicate unstimulated cells. Similar results were obtained on three separate occasions.
DISCUSSION

Chapter 3 described the purification, identification and characterisation of a Rac1/RhoGDI complex which can retard the secretory rundown of permeabilised mast cells. The intact heterodimer is necessary: by itself, the Rac protein is without effect, while RhoGDI by itself accelerates the rate of rundown. This chapter describes the purification of recombinant Rac2 (rRac2) from E.Coli and its application in the rundown assay. As discussed in the previous section, the purification proved to be less straightforward than anticipated.

The first problem that I encountered was that some of the transformed bacterial cells were producing protein that migrated as distinct bands approximately 1-2 kDa apart on SDS-PAGE (shown in Figure 4.2). The appearance of this doublet was most likely due to proteolytic degradation occurring within the bacterial cells. This idea is supported by the finding that the homogeneous protein preparations produced by some of the cells (which were eventually selected for subsequent purifications of rRac2) consisted entirely of the upper of the two bands observed on a polyacrylamide gel (i.e. the larger one). It is already established that bacterial cells subjected to overexpression regimes can recognise excesses of foreign protein and in some cases, these will be selected for proteolysis (Brown, 1990). I assume that this was what occurred here. "Selecting" bacteria in the manner described has presumably isolated cells which do not exhibit such high levels of protease activity. Having made this point, the presence of two forms of rRac2 made little difference to the activity of the protein in the rundown assay (Figure 4.6b). However, as the exact nature of the difference between the two forms of the protein was unknown I thought it best to use a single form of protein throughout.

rRac2 was without effect in the rundown assay unless it was preactivated by binding GTPγS (Figures 4.8 and 4.9). In this way it is quite different from the wild-type protein, which must be presented as its complex with RhoGDI. It is likely that the differences are due to the lack of post-translational modifications on the recombinant protein without which it fails to interact with RhoGDI, fails to translocate to the plasma membrane (Abo et al., 1994; el Benna et al., 1994;
Quinn et al., 1993; Didsbury et al., 1990; Heyworth et al., 1994) and fails to interact with some soluble and membrane bound exchange factors (Ando et al., 1992; Heyworth et al., 1993; Takai et al., 1993; Bokoch et al., 1994).

As shown in Figure 4.9, the GTPγS-bound (preactivated) rRac2 retards the rundown of the secretory response to the dual stimulus (Ca²⁺ plus GTPγS) in permeabilised mast cells in a similar manner to the purified Rac1/RhoGDI complex FOAD II. This result nicely confirms the validity of the results obtained previously (Chapter 3). Unlike the FOAD II, however, preactivated rRac2 exerts its effects by interacting directly with its downstream effector(s) i.e. without the involvement of exchange factors.

The most exciting finding was that, in the presence of preactivated rRac2, secretion could now be stimulated in the absence of GTPγS i.e. using only Ca²⁺ as the stimulus. Previously it had been shown that mast cells can be stimulated to secrete by GTPγS in the effective absence of Ca²⁺ (Fernandez et al., 1984; Lillie and Gomperts, 1992b; Larbi and Gomperts, 1996), but never before had it been possible to induce secretion in the absence of a stimulating guanine nucleotide (Lillie and Gomperts, 1992b). This activity provides additional evidence that Rac is a normal regulator of mast cell secretion in vivo. However, an obvious concern here must be the possibility that this GTPγS-independent secretion might not be due to the protein but to the presence of free nucleotide leaking from the preactivated rRac2, especially since it becomes apparent almost immediately after permeabilisation, before the protein would have had time to penetrate the cells. It should be emphasised that in these experiments, following addition of the stimulus, a 20 minute period was always allowed in order to ensure that the exocytotic process (always slow in rundown cells) would proceed to completion. This would also have allowed plenty of time for the exogenous protein to enter the cells and exert an effect in conjunction with the Ca²⁺ added previously. There are several reasons for thinking that this is indeed what actually happens:

1. Following prebinding of GTPγS in a low Mg²⁺ environment, the protein was passed through a desalting column to exchange the buffer to one of high Mg²⁺ (suitable for use in secretion assays) and also to remove all free nucleotide (>99.9%, as assessed by measurements of
A280). This buffer exchange operation takes about 20 minutes, so since it has been demonstrated that nucleotide dissociation from Rac (in the presence of excess free nucleotide) occurs in the first ten minutes following binding (Self and Hall, 1995a), then any dissociation would be expected to be complete before the protein was applied to the cells and the high Mg2+ buffer would also have retarded any further detachment of bound nucleotide. In addition, the virtual absence of free nucleotide in the protein solution following the desalting procedure, would make it more likely that the protein would retain its bound nucleotide since there would be no nucleotide to replace that lost, and it is known that Rho proteins are unstable in the absence of bound nucleotide (Self and Hall, 1995b).

2) In 3 out of 4 experiments, the extent of secretion declined as the concentration of preactivated rRac2 was increased above an optimum.

3) Other Rho-related proteins, in particular recombinant F37A-Racl loaded with GTPyS and treated identically as in the experiments reported here, failed to induce secretion when introduced into mast cells at similar concentrations to those tested here (<1% release: A. J. O'Sullivan, unpublished observations). This indicates that if any nucleotide leakage is occurring, then it does so to an extent quite insufficient to stimulate secretion.

I am confident that the results represent the specific actions of rRac2. However, it does not necessarily follow that Rac2 is the native GTP-binding protein (G_E) mediating exocytosis in normally stimulated intact cells. The demonstration that Rac is normally present in these cells, and also that it leaks following permeabilisation, is certainly consistent with this idea (Brown et al., 1997) but it is also possible that it can substitute for other endogenous regulator(s) which might have leaked or become inactivated under these conditions. Another interesting observation is that for cells supported by preactivated rRac2, application of the dual stimulus (Ca2+ plus GTPyS) then elicits a further increment of secretion, above that stimulated with Ca2+ alone (Figure 4.9). This suggests the involvement of at least one additional GTP-binding protein in the regulation of secretion. As
candidates, we should consider the heterotrimer $G_{13}$ (Aridor et al., 1993), and Rho (Price et al., 1995).

The reasons for using Rac2 (rather than rac1) in these experiments were outlined in the introduction to this chapter. At first glance, it appears that rRac2 is indeed more active than rRac1, previously shown by others to stimulate secretion in permeabilised mast cells (Price et al., 1995). This might be to differences in experimental procedure. The rRac1 was tested in cells which had been extensively washed following permeabilisation (to ensure complete removal of soluble proteins) and involved the use of the dominant acting V12Racl mutant. Not only is this GTPase deficient, but it also exhibits a reduced level of effector binding compared to the wild type protein. While the GTPase deficient mutant and the preactivated wild type protein might be equivalent in some respects, they are obviously very different in others. There is also the possibility that they may exhibit differences in folding attributable to the mutation which could affect activity in other as yet undefined ways. It is for these reasons that wild type proteins were used for most of the experiments presented here. However, recent experiments now indicate that rRac1 is as effective as rRac2 when tested under the conditions described here (A. J. O'Sullivan, unpublished observations).

It has not been established whether Rac1 and Rac2 can exert equal effects on the same effectors, or whether they address unique effectors. Very little work has so far been carried out using Rac2, despite this being the most abundant form in some cell types (Didsbury et al., 1989; Reibel et al., 1991). The exception is the study of the NADPH oxidase of phagocytic cells for which Rac1 is an essential component of the active enzyme complex in guinea pig macrophages (Abo et al., 1991; Pick et al., 1993), whereas Rac2 is essential in human neutrophils (Knaus et al., 1991; Kwong et al., 1993). Of course, these conflicting results may simply reflect species differences, but it has since been found that in a total reconstitution of the NADPH oxidase system from recombinant proteins Rac1 and Rac2 are equally effective (provided that they are post-translationally modified) (Ando et al., 1992; Heyworth et al., 1993). However, in the presence of cytosol, Rac2 becomes more effective than Rac1, which might indicate the action of a cytosolic factor capable of suppressing Rac1 activity (Heyworth et al., 1993). This agrees with the finding that Rac2 interacts much more strongly than Rac1 with its target
protein in the NADPH oxidase, p67phox, using a yeast two-hybrid system to detect the interaction where once again, it is possible that factors are present in the yeast cell that suppress Rac1 interaction with p67phox (Dorseuil et al., 1996).

From all this one can conclude that whilst both forms of Rac are capable of stimulating activity in vitro, regulatory mechanisms may exist in vivo that result in Rac2 predominating in the regulation of the NADPH oxidase. Attempts to identify the region of Rac involved in the activation of the oxidase have offered few insights, revealing evidence implicating both the N-terminal (amino acid sequences common to both forms of the protein) and the C-terminal regions (which are divergent) (Diekmann et al., 1995; Dorseuil et al., 1996; Freeman et al., 1994; Kwong et al., 1995). However, in human cells at least, the greater abundance of Rac2 in neutrophils (Didsbury et al., 1989; Abo et al., 1994; Heyworth et al., 1994; Reibel et al., 1991) makes it likely that it is this form which delivers the normal stimulus. It remains to be seen whether there are species differences (as between humans and guinea pigs (Abo et al., 1991; Pick et al., 1993)), or whether other Rac-regulated functions are regulated predominantly by one form or the other.

In the present experiments, rRacl and rRac2, tested under identical conditions, are equally active in the support of secretion so both forms are at least equally capable of acting as regulators (at least in the permeabilised cells). However, as with the neutrophil NADPH oxidase, this may not actual situation that pertains in intact cells. Due to the relative abundance of Rac2 in myeloid cells (Didsbury et al., 1989) must be likely that Rac2 is the actual biological regulator.

My experiments using both Rac1 (as FOAD II) and preactivated rRac2, presented individually and together (Figure 4.11) were carried out to compare their effects, and also to determine whether they interact with common or distinct effectors. It should be stressed that an optimal amount of preactivated rRac2 was used, whilst a supra-optimal amount of FOAD II was used. As expected, when stimulated by Ca2+ plus GTPγS (Figure 4.11b), both proteins enhanced the level of secretion. One might have expected that if they address the same effector and with equal efficiency, then an optimal dose would stimulate the cells to secrete to a similar extent. Instead, the effect of FOAD II is considerably greater than that of rRac2.
As discussed earlier (Chapter 3, Discussion), the Rac1, coupled to RhoGDI, is probably translocated to the plasma membrane (either before or after nucleotide exchange), to become localised for interaction with exchange factors and/or effector proteins. This may promote the accumulation of high local concentrations of effector, resulting in optimal stimulation of secretion. In contrast, the non-post-translationally modified recombinant Rac2 cannot undergo such membrane localisation and this may reduce the efficiency of its interaction with effector proteins, resulting in lower levels of secretion. There is also the possibility that while Rac1 presented as the purified FOAD II complex can interact with both cytosolic and membrane bound effectors the actions of rRac2 are restricted to effectors in the cytosol.

It may be that Rac1 can interact with effectors that Rac2 cannot. This is an interesting possibility, since Rac1 can interact with effectors of Cdc42Hs (the human homologue of the yeast cell cycle protein), another member of the Rho family of GTPases. Examples of these common effectors include p21^Cdc42/Rac1-activated protein kinase (PAK) (Manser et al., 1994; Manser et al., 1995; Teo et al., 1995; Prigmore et al., 1995), PI-3 kinase (Tolias et al., 1995; Bokoch et al., 1996), 70kDa ribosomal S6 kinase (pp70S6k) (Chou and Blenis, 1996), and proteins containing the Cdc42/Rac interactive binding (CRIB) region (Burbelo et al., 1995). Also, Cdc42 and Rac have been shown to exert regulatory effects on the same processes. An example is the activation of c-Jun amino-terminal kinase (JNK) (Coso et al., 1995; Zhang et al., 1995; Bagrodia et al., 1995; Collins et al., 1996) though it is not known whether these effectors distinguish between Rac1 and Rac2. Obviously, it is possible that the effects of the FOAD II complex (and possibly those of rRac2) on secretion occur as a result of interactions, not with Rac effectors, but with Cdc42 effectors. In this case, the exogenous Rac would be substituting for the function of leaked Cdc42.

When both proteins are presented together, there is certainly an increase in the extent of secretion that can be induced by Ca^{2+} plus GTPγS over that which occurs when the proteins are presented singly (Figure 4.1b). Although this appears small, it occurs against an already high level of secretion when such increments obviously become harder to achieve. If, as already postulated, there is more than one effector for Rac, this may be indicative of some sort of co-operation between
them. Alternatively, it may be that the enhancement due to the rRac2 occurs because, being preactivated, it can interact more swiftly with its effectors (especially those present in the cytosol) than the Rac1 in its complex with RhoGDI. Under normal conditions, cytosolic effectors may translocate to the membrane in order to interact with the native Rac, and the use of preactivated rRac2 could negate the need for this step. Of course, if the accumulation of Rac and effector proteins at common sites in the membrane is necessary for optimal activity as already discussed, then this may only be of limited advantage. Hence this may be why the increase in secretion seen under these conditions with both proteins is consistently quite small.

The conclusions to be drawn from the experiment shown in Figure 4.11a also indicate that regulation of secretion by Rac may not be quite so simple as first appears (!). While preactivated rRac2 supports substantial secretion (31%) in response to Ca^{2+} alone, FOAD II supports only a few per cent (5%). However, there is a considerable increase in secretion when both proteins are presented together. The proteins are exhibiting distinct synergy (i.e. 31+5 = 58) in the regulation of mast cell secretion under these conditions. When it is considered that there was no added GTPγS which could have activated the Rac1 component of the FOAD II complex, this result is most surprising. Even if GTPγS were leaking off the rRac2, there could not have been sufficient to activate the FOAD II optimally. In these experiments, the FOAD II was provided at a supra-optimum concentration and so, according to the equilibrium proposal for Rho protein activation/inactivation presented earlier (Chapter 3, “Discussion”), there may have been sufficient Rac1 available to ensure some membrane localisation and activation of effectors even in the absence of an activating nucleotide. The results shown in Figure 4.11b have already provided evidence that there may be more than one effector for Rac, so the Rac1 may be interacting with the aforementioned membrane bound effectors with which rRac2 cannot efficiently interact. Coupled to this, if there is some kind of synergistic relationship between the Rac effectors, then this could explain why the increase in secretion which occurs as a result of incubation with both proteins is so large.
Further evidence for the involvement of Rac in the regulation of mast cell secretion was sought by testing the effect of the dominant negative mutant rT17N-Rac2. The analogous mutant of Ras forms stable inactive complexes with exchange factors (Quilliam et al., 1994) and also possesses preferential affinity for GDP owing to improper co-ordination of Mg$^{2+}$ (Farnsworth and Feig, 1991). Purification of this protein proved very difficult. It was insoluble under certain conditions (low salt, high pH) and adhered strongly to most surfaces. The failure to achieve gel filtration was, with hindsight, probably due not to its irreversible adsorption to the gel matrix, but to its adherence to the membrane used in the previous concentration step with the consequence that no protein was loaded onto the column in the first place! As the rT17N-Rac2 protein is so adhesive, it is probable that the concentrations given in Figure 4.19a represent an overestimate of the actual concentrations delivered to the cells in the assay. However, inhibition of mast cell secretion by the rT17N-Rac2 is clearly apparent, implying the presence of an exchange factor recognising Rac which is involved in the regulation of exocytosis.

In conclusion, the data showing stimulation of secretion by preactivated rRac2 and showing inhibition of secretion by rT17N-Rac2 give a strong indication that Rac2 is a regulator of secretion in mast cells. The mechanisms by which it exerts its effects remain to be determined. An obvious candidate would be via rearrangements of the cytoskeleton shown to be mediated by Rac (and Rho) in mast cells (Norman et al., 1994), since such rearrangements have generally been considered necessary for secretion to occur (reviewed in (Trifaro and Vitale, 1993)). However, it was recently shown that secretion induced by Rac (and Rho) can proceed unhindered even when cytoskeletal rearrangements are blocked, suggesting an alternative mechanism whereby Rac regulates mast cell secretion (Norman et al., 1996)(Sullivan & Koffer, manuscript submitted). There is increasing evidence showing that Rac can act downstream of Ras, for example in the activation of c-Jun amino terminal kinase (JNK) (Khosravi Far et al., 1995; Coso et al., 1996; Collins et al., 1996), and also that Ras and Rac/Rho signalling pathways are associated through the interactions between Ras-GAP and p190 Rho-GAP (Settleman et al., 1992; McGlade et al., 1993). However, although Ras has been found to induce degranulation (the morphological counterpart of secretion) of mast cells, it only does so following a prolonged period (4 hours).
Bar-Sagi and Gomperts, 1988) and its effects are most probably indirect. Secretion is, of course, an abrupt process and most of these descriptions of Ras-Rac interaction refer to processes of much longer duration. What is clear is that Rac can regulate a diversity of cell functions, maybe by interacting with effectors unique to each pathway (Lamarche et al., 1996; Joneson et al., 1996; Westwick et al., 1997). It has also been suggested that Rac interacts with each of its effectors via different regions (i.e. different combinations of the same regions) in its structure (Freeman et al., 1996).

Candidate effector proteins for Rac in the regulation of mast cell secretion must include the p21$^{\text{Cdc42/Rac1}}$-activated kinases (PAKS) (Manser et al., 1994; Manser et al., 1995; Teo et al., 1995; Prigmore et al., 1995), PI-3kinase (Tolias et al., 1995; Bokoch et al., 1996), and proteins containing the Cdc42/Rac interactive binding (CRIB) region (Burbelo et al., 1995), although it is quite possible that the actual effectors will eventually be found to be unique to secretory regulation. Their identity remains to be determined.

To summarise, in this chapter I have presented data supporting ideas initially presented in Chapter 3, suggesting a role for Rac as a regulator of mast cell secretion. In addition, I have presented data indicating that other GTPases are also involved, in particular hinting at the possible involvement of Cdc42, another member of the Rho family.
CHAPTER 5

"PURIFICATION AND CHARACTERISATION OF RECOMBINANT CDC42Hs AS A REGULATOR OF EXOCYTOSIS"
INTRODUCTION

The demonstration that Rac (chapter 3) and Rho (Price et al., 1995; Norman et al., 1996), can act as regulators of secretion in mast cells gives a strong indication that GTPases of the Rho family in general may be the biological regulators of this process. These proteins also regulate other diverse cellular functions, including re-organisations of the actin cytoskeleton (Nobes and Hall, 1995; Kozma et al., 1995), integrin-mediated signalling pathways (Parsons, 1996; Hotchin and Hall, 1995), receptor-mediated endocytosis (Lamaze et al., 1996), activation of transcription (Hill et al., 1995), and oogenesis in Drosophila (Murphy and Montell, 1996).

In particular, Rac1 and Cdc42 have been shown to interact with several common effectors, examples of which have already been mentioned. Experiments presented in Chapter 4 gave a hint that Cdc42 may be involved in secretory regulation, as are Rac and Rho. I am aware of only two studies investigating the role of Cdc42 in exocytosis, (see Chapter 1, Introduction). One suggests that carboxylmethylation of Cdc42 is involved regulation of insulin secretion from pancreatic islets and β-cells (Kowluru et al., 1996) while the other finds no role for this protein as a regulator of secretion in PC12 cells (Komuro et al., 1996b).

This chapter describes the purification of recombinant Cdc42Hs and its dominant negative T17N mutant from E.Coli and their characterisation as regulators of mast cell secretion. Also described are studies carried out to determine whether Cdc42 is present in mast cells, and whether it leaks from these cells upon permeabilisation with SL-O.
RESULTS

Purification of rCdc42Hs

Recombinant Cdc42Hs (rCdc42Hs) was expressed in *E.Coli* as the GST-fusion protein, and purified using glutathione Sepharose followed by thrombin cleavage according to a slight modification of a published method (Self and Hall, 1995b). No problems were encountered, and SDS-PAGE analysis of samples taken at various stages are shown in Figure 5.1. As can be seen, the appearance of the gels is almost identical to those of the Rac2 purification procedures, with the fusion protein apparent in bacterial pellets and supernatants (Figure 5.1a, lanes CP1, CS1 and CP2, CS2), which is bound and then cleaved from the glutathione Sepharose beads (Figure 5.1b, lanes ptS1, ptS2). Gel analysis of the final protein product, following gel filtration, shows a gross band of pure rCdc42Hs, with some evidence of proteolysis. It migrates at approximately 24 kDa (lane pG75). Note that the band evident in the same lane at approximately 45 kDa is probably due to contamination by the gross band of fusion protein in the adjacent lanes ptwB1 and ptwB2, which contain samples of beads following thrombin treatment and washing. It is possible that the efficiency of thrombin cleavage could have been improved by, for example, increasing either the concentration of thrombin or the time of thrombin treatment, but due to time limitations, this was not attempted.

As with rRac2, and in accordance with previous findings (Self and Hall, 1995b), it was found that approximately 10% of the total purified protein was capable of binding guanine nucleotide, and this was taken to be the concentration of active protein.

Effect of rCdc42Hs on mast cell secretion

Similar to Rac, preactivated (GTP\_S bound) rCdc42Hs retards the rate of secretory run down of permeabilised mast cells. Again, after allowing time for the preactivated protein to penetrate the permeabilised cells, it is possible to elicit secretion by application of Ca\(^{2+}\) alone. In the experiment illustrated in Figure 5.2a (filled squares), the effect of rCdc42Hs first became apparent when applied to
**Figure 5.1 Purification of recombinant Cdc42Hs (rCdc42Hs)**

*E. Coli* transformed with cDNA encoding Cdc42Hs-GST were grown overnight at 37°C with agitation and after dilution, grown for a further 1 hour. Protein production was then induced by addition of 100μM IPTG and the cells grown for a further 3 hours. Cells were harvested by centrifugation, resuspended in Digestion buffer plus protease inhibitors (see Chapter 2) and disrupted by sonication after which the lysate was centrifuged to obtain cytosolic and membrane fractions. The supernatant was then incubated with glutathione Sepharose for 1 hour at room temperature after which the beads were washed. rCdc42Hs was cleaved from the beads by thrombin (~5 U.ml⁻¹) for 1 hour at room temperature after which the free protein was separated from the beads by centrifugation. Thrombin was removed by incubation with p-aminobenzamidine agarose and the protein was then concentrated to 2ml and applied to a G75 Superdex (gel filtration) column (HiLoad 16/60, preparation grade, XK16, volume 122ml), which was pre-equilibrated in Digestion buffer plus protease inhibitors as detailed in Chapter 2. The column was eluted using this buffer and 800μl fractions collected.

Samples taken throughout this procedure were treated with sample buffer before being separated on 12% polyacrylamide gels. Proteins were visualised by silver staining.
Figure 5.2 Concentration effect relationship for the enhancement of secretion by preactivated rCdc42Hs for stimulation by Ca\(^{2+}\) alone and by Ca\(^{2+}\) plus GTP\(_\gamma\)S.

a) Mast cells were permeabilised by treatment with streptolysin-O at 0°C for 5 min, washed and then brought to 37°C in Rundown buffer (pH6.8) containing Mg,ATP (1mM) and sufficient EGTA (0.1mM) to suppress Ca\(^{2+}\) to below pCa8. Cells were allowed to run down for 7 minutes (Ca\(^{2+}\) stimulus) and 17min (Ca\(^{2+}\) plus GTP\(_\gamma\)S) before transfer to solutions containing Ca,EGTA (3mM) to regulate pCa5 (or pCa7 for non-stimulated control cells) in the presence or absence of GTP\(_\gamma\)S (100µM). The incubation was continued for a further 20min, at which time the cells were sedimented by centrifugation and the supernatant assayed for the presence of secreted hexosaminidase. Results are expressed as means ± SEM (n=4), error bars are omitted where too small for inclusion. ■, Cells stimulated with Ca\(^{2+}\) (pCa5) alone; △,cells stimulated by Ca\(^{2+}\) plus GTP\(_\gamma\)S (100µM); ○, unstimulated cells. Similar results were obtained on at least three separate occasions.

b) Data presented as Hill plots. For secretion stimulated by Ca\(^{2+}\) alone, slope \(p = 0.9\) (\(r = 0.98\), n = 5 data points); for secretion stimulated by Ca\(^{2+}\) plus GTP\(_\gamma\)S, \(p = 0.6\) (\(r = 0.97\), n = 8 data points). Analysis of four experiments indicates that the Hill slopes for stimulation by Ca\(^{2+}\) alone and by Ca\(^{2+}\) plus GTP\(_\gamma\)S are not significantly different.
permeabilised cells at about 0.066 μg.ml⁻¹ (3.1nM, assuming MW = 21.3kDa (Shinjo et al., 1990)) and the extent of secretion was then progressively enhanced as the concentration of the protein was increased to 0.53 μg.ml⁻¹ (24.9nM) and even above this. Unlike the response to Rac2 which is confined within a single decade range of protein concentration, the response to preactivated rCdc42Hs is extended over a wider range, approaching two decades. The responses to preactivated rCdc42Hs in run-down mast cells stimulated by the dual stimulus (Ca²⁺ plus GTPγS) were also measured (Figure 5.2a). The results of four experiments are summarised in Table 5.1. It can be seen that, as with Rac2 (Figure 4.10), provision of free GTPγS again enhances the extent of secretion over the level achieved by Ca²⁺ alone, but in addition, it has the effect of increasing the sensitivity to rCdc42Hs approximately 6-fold. The extended concentration range of the response to rCdc42Hs (as indicated by the Hill coefficient) appears to be unaltered by the presence of free GTPγS. Clearly regulation by Rac and Cdc42 in the regulatory process leading to secretion is complex and the differences between them are subtle.

Table 5.1  Summary of data obtained from studies of the concentration-effect relationship of rCdc42Hs in the enhancement of mast cell secretion stimulated by Ca²⁺ or Ca²⁺ plus GTPγS

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>mean EC₅₀ (μg.ml⁻¹)</th>
<th>SEM (n=4 expts)</th>
<th>diff (p)</th>
<th>mean Hill Coeff</th>
<th>SEM (n=4 expts)</th>
<th>diff(p)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca²⁺</td>
<td>0.42 (19.7nM)</td>
<td>0.068</td>
<td>0.0047</td>
<td>1.07</td>
<td>0.15</td>
<td>0.12</td>
</tr>
<tr>
<td>Ca²⁺ + GTPγS</td>
<td>0.064 (3nM)</td>
<td>0.045</td>
<td>0.73</td>
<td>0.11</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

After carrying out similar experiments, testing the effect of different rCdc42Hs preparations on the secretory response to Ca²⁺ (the response to the dual stimulus was not further investigated), it became apparent the concentrations supporting secretion were subject to some variation, although the extended (two decade) range of concentrations of preactivated rCdc42Hs activating secretion was always
Figure 5.3  Saturated concentration effect relationship for the enhancement of secretion by preactivated rCdc42Hs

Purified mast cells were treated as detailed in the legend to Figure 5.2, using rCdc42Hs from a different batch, and allowed to run down for 7 min before stimulation with Ca\(^{2+}\) (pCa5). Results are expressed as means ± SEM (n=4), error bars are omitted where too small for inclusion. Closed symbols indicate cells stimulated with Ca\(^{2+}\) (pCa5) alone, open symbols indicate unstimulated cells. Similar results were obtained on at least three separate occasions.
observed. In addition, in some experiments (for an example, see Figure 5.2a) the response failed to saturate while in others saturation was clearly achieved (Figure 5.3). These differences were certainly greater than might be expected even taking into account the vagaries of the rundown assay, and appeared to be determined by variation between the batches of protein. In order to pinpoint any differences, samples of each batch were retained and they were analysed together by SDS-PAGE, as shown in Figure 5.4. This revealed the presence of two or more polypeptides (on one occasion four) running close together, possibly indicating the action of bacterial endopeptidases. In order to protect against this, I decided to use a recombinant C-Myc tagged Cdc42Hs (rC-Myc-Cdc42Hs) for all subsequent experiments in the hope that any endopeptidase activity would be restricted to the C-terminal tag, leaving the GTPases intact.

**Purification of rC-Myc-Cdc42Hs**

Purification of the rC-Myc-tagged Cdc42Hs (again using the GST-fusion method) was achieved without difficulty, according to a modification of the published method (Self and Hall, 1995b). The progress of the purification, as analysed by SDS-PAGE, is shown in Figure 5.5. The majority of the GST-fusion protein, migrating at approximately 45kDa, was soluble (Figure 5.5a, lane CS), and after binding to glutathione Sepharose beads, a large quantity of rC-Myc-Cdc42Hs was cleaved from the beads by treatment with thrombin (Figure 5.5b, lanes ptS). Washing removed most of this protein from the beads (compare Figure 5.5b, lanes ptB and ptwB), and analysis of the final purified product following gel filtration revealed only a single band at approximately 25kDa (i.e. slightly larger than non-tagged rCdc42Hs, as would be expected), shown in Figure 5.5b, lane pG75. Measurements of guanine nucleotide binding indicated that approximately 20% of this protein was active.

**Effect of rC-Myc-Cdc42Hs on mast cell secretion - can Cdc42 interact with a Rac effector in the regulation of secretion?**

As shown in Figure 5.6, the tagged rCdc42Hs protein again enhances secretion, and moreover, the sensitivity to stimulation again extends over a two decade range of concentration (open symbols) indicating that the Myc-tag has little or no
Figure 5.4 SDS-PAGE analysis of four separate batches of rCdc42Hs

Samples from four separate batches of purified rCdc42Hs (pG75/1-pG75/4) were treated with sample buffer before being separated on 12% polyacrylamide gels. Proteins were visualised by silver staining. In each case, more than one polypeptide was apparent, indicative of proteolysis.
Figure 5.5 Purification of recombinant C-Myc-Cdc42Hs (rC-Myc-Cdc42Hs)

E.Coli transformed with cDNA encoding C-Myc-Cdc42Hs-GST were grown (in LB broth) and harvested and the membrane and supernatant fractions separated as detailed in the legend to Figure 5.1, (except that the bacteria were grown for 4 hours following induction and the buffer used here was buffer “A” - see Chapter 2). The supernatant (supplemented with 15mM DTT) was dialysed against buffer A plus 0.1mM DTT before being incubated with glutathione Sepharose for 30min at 4°C. The beads were washed and then rC-Myc-Cdc42Hs was cleaved by thrombin (10U.ml bed volume⁻¹) overnight at 4°C following which half the previous amount of thrombin was added and cleavage continued for an extra 2 hours. Purified protein was then separated from the beads by centrifugation. Thrombin was removed by incubation with p-aminobenzamidine agarose and the protein was then concentrated to 2ml and applied to a G75 Superdex (gel filtration) column (HiLoad 16/60, preparation grade, XK16, volume 122ml), which was pre-equilibrated and eluted in modified buffer A (see Chapter 2 for details) as in the legend to Figure 5.1.

Samples taken throughout this procedure were treated with sample buffer before being separated on 12% polyacrylamide gels. Proteins were visualised by silver staining.
Figure 5.6  Concentration-effect relationships for preactivated rC-Myc-Cdc42Hs applied either alone, or together with a saturating concentration of preactivated rRac2 (1μg ml⁻¹).

Cells were permeabilised as in the legend to Figure 5.2 and allowed to run down for 7 minutes in the presence of proteins before stimulation with Ca²⁺ (pCa5). % secretion values indicate stimulated release above control (non-stimulated cells) expressed as a percentage of total cellular hexosaminidase (see Chapter 2 for details). Results are expressed as means ± SEM. (n=4), error bars are omitted where too small for inclusion. Open symbols indicate cells incubated with rC-Myc-Cdc42Hs alone; closed symbols indicate cells incubated with rRac2 (1μg ml⁻¹ active protein) and rC-Myc-Cdc42Hs together. Similar results were obtained on three separate occasions.
effect on the ability of this protein to act as a regulator. Also, using the tagged protein, saturation of the stimulated responses was consistently achieved. In the experiment illustrated the enhancement of secretion extended over a range of concentrations from about 0.11 μg.ml⁻¹ to 3.5 μg.ml⁻¹ (EC₅₀~0.3 μg.ml⁻¹, 13.5nM, as drawn [assuming MW of C-Myc tag to be approximately 1kDa, from estimates of MW differences using SDS-PAGE]) inducing a maximum of 65% secretion.

With the aim of determining whether the two GTPases, Cdc42 and Rac can interact with a common effector in the stimulation of secretion, as suggested in the previous chapter, rC-Myc-Cdc42Hs was titrated in the run-down experiment in the presence of a saturating concentration of rRac2. Preactivated rRac2, applied at a concentration above its optimum (1μg.ml⁻¹ active protein) in the absence of rC-Myc-Cdc42Hs induced 40% secretion in response to the Ca²⁺-only stimulus. When rC-Myc-Cdc42Hs was introduced at concentrations in the low end of its activating range (0.1-0.9 μg.ml⁻¹) together with the saturating concentration of rRac2, there was a further enhancement of secretion above that supported by rC-Myc-Cdc42Hs applied alone. As the concentration of rC-Myc-Cdc42Hs was increased above 0.9 μg.ml⁻¹, the simultaneous presence of the rRac2 was without apparent effect, with the extent of secretion about the same as that induced by rC-Myc-Cdc42Hs alone. As can be seen, even in the presence of a saturating concentration of rRac2, the activating effect of rC-Myc-Cdc42Hs is still expressed throughout its normal two decade concentration range, the only difference being the high baseline secretion of 40% due to the presence of the rRac2. The results of this experiment are consistent with the idea that there may be two downstream effectors with differing affinities for rC-Myc-Cdc42Hs, one of which (the one with lower affinity for rC-Myc-Cdc42Hs) can also be activated by rRac2. In other words, both rRac2 and rC-Myc-Cdc42Hs can exert regulatory effects on secretion by acting on a common effector, but rC-Myc-Cdc42Hs can also exert an effect through a higher affinity interaction with an additional effector, with which rRac2 does not interact.

**Purification of rT17N—Cdc42Hs**

In an attempt to provide further evidence of a role for Cdc42 in the regulation of mast cell secretion, the recombinant T17N dominant negative mutant form was
purified for use in secretion experiments in a similar manner to the rT17N-Rac2 described in Chapter 4.

As before, the mutant Cdc42Hs was expressed in *E.Coli* as a GST fusion protein and purified according to a slight modification of the published method (Self and Hall, 1995b). In spite of the close resemblance between the two proteins (Rac and Cdc42Hs are 70% homologous, and 36 out of the first 40 amino acids at the amino terminus are identical (Shinjo et al., 1990)) the rT17N—Cdc42Hs proved easy to purify, and did not exhibit the adhesive properties shown by the rT17N-Rac2. Unfortunately the yield of protein was low (typically 200 μg.l⁻¹ of *E.Coli* suspension). SDS-PAGE analysis of samples taken throughout the purification is shown in Figure 5.7. A large amount of fusion protein is apparent in the bacterial supernatant (lane CS, at approximately 45kDa), this is bound to the beads and the rT17N—Cdc42Hs was released by thrombin cleavage (lane ptS). The majority of the cleaved protein was removed from the beads by washing (see lane ptwS and compare lanes ptB and ptwB), although a considerable proportion of the fusion protein remained uncleaved (lane ptwB). The efficiency of cleavage could probably be improved, but this was not investigated further. Analysis of the final product following gel filtration revealed a single discrete band migrating at approximately 24kDa, as would be expected (lane pG75).

As with rT17N-Rac2 (see Chapter 3), in three separate experiments (six replicate determinations in each), no significant [³H]GDP binding by this protein could be detected (data not shown). The reasons for the apparent lack of nucleotide binding by these mutant proteins are unknown, and were not investigated further.

**Effect of rT17N—Cdc42Hs on mast cell secretion**

The experiment illustrated in Figure 5.8 demonstrates the effects of rT17N-Cdc42Hs on secretion from permeabilised mast cells. Since the extent of nucleotide binding of this protein could not be assessed, the concentrations discussed and recorded in Figure 5.8 represent totals, not active protein concentrations. In these experiments, the cells were allowed to run down briefly, (7 minutes), sufficient to allow the protein to penetrate the cells whilst ensuring a high level of release, so that any inhibition should be clearly evident. Secretion
Figure 5.7 Purification of recombinant T17N-Cdc42Hs (rT17N-Cdc42Hs)

*E. Coli* transformed with cDNA encoding T17N-Cdc42Hs-GST were grown (in LB broth), harvested, and the rT17N-Cdc42Hs purified exactly as in the legend to Figure 5.5.

Samples taken throughout this procedure were treated with sample buffer before being separated on 12% polyacrylamide gels. Proteins were visualised by silver staining.
Figure 5.8 Inhibition of GTPγS stimulated secretion by rTN17-Cdc42Hs.

Cells were permeabilised as in the legend to Figure 5.2 and allowed to run down for 7 minutes before stimulation with Ca²⁺ (pCa5) and GTPγS (100μM). Results are expressed as means ± SEM (n=4), error bars are omitted where too small for inclusion. Closed symbols indicate stimulated cells, open symbols indicate unstimulated cells. Similar results were obtained on three separate occasions.
Figure 5.9 Leakage of Cdc42 from SL-O permeabilised mast cells

Cells, pretreated with diisopropylphosphofluoridate to prevent proteolysis were treated as detailed in the legend to figure 5.2 and timed samples were removed as indicated. The cells were sedimented and the proteins present in the external salts solution precipitated with acetone at -20°C. They were then separated by SDS-PAGE on a 12% gel and transferred to nitrocellulose using a wet blot system (BioRad) run at 250mA constant current for 1 hour. The proteins were then probed with an antibody to Cdc42Hs and immunoreactivity was detected by the ECL technique. The figure shows the appearance of the ECL film.
was then induced by application of the dual stimulus (Ca$$^{2+}$$ plus GTPγS). Due to limited protein availability the highest (total) concentration that could be applied was 50 µg.ml$$^{-1}$$. In the experiment illustrated, this concentration of protein caused 20% inhibition of secretion ($p<0.002$, $n=4$) and in two other experiments, the highest concentrations of rT17N—Cdc42Hs tested caused 10% and 20% inhibition.

Is Cdc42 present in mast cells?

The data shown so far present a strong case for Cdc42 being a biological regulator of mast cell secretion. However, it remains possible that rCdc42Hs is merely capable of substituting for another regulator, and does not perform this role physiologically. Supporting evidence for a role for Cdc42 as a true regulator of mast cell secretion would include the demonstration that it is present in mast cells, and that it leaks under the conditions normally used in the rundown experiments. To this end, the leakage of Cdc42 was assessed, analysing for the presence of the protein in the extracellular fluid at intervals following permeabilisation, as previously described to detect the leakage of RhoGDI (Chapter 3, figure 3.7). Analysis of leaked proteins by SDS-PAGE and Western blotting (Figure 5.9) revealed the presence of Cdc42 as a band running at approximately 24 kDa as expected. Unfortunately, despite repeated attempts, it was never possible to obtain a clearer result than that presented here. However, the presence of Cdc42 in mast cells is established, and this is seen to leak rapidly from the cells upon permeabilisation.

To summarise, data presented in this chapter reveal a role for Cdc42 as a regulator of mast cell secretion. It appears to exert its effects through interactions with more than one effector, one of which can also be activated by Rac, already shown to be a regulator of mast cell secretion.
DISCUSSION

The results of the experiments presented in the previous section show that rCdc42Hs can stimulate secretion when introduced into mast cells in its preactivated state (Figure 5.2). As with Rac, secretion can be stimulated by a Ca\(^{2+}\)-only stimulus (in the absence of exogenous GTP\(\gamma\)S) and can be further enhanced if GTP\(\gamma\)S is also supplied (dual stimulus). These findings disagree with those of the only other study so far designed to test for a direct role of the Cdc42 protein in regulated secretion, which found no evidence for such a role in PC12 cells (Komuro et al., 1996b). This may be due to differences in experimental approach since this investigation employed a co-transfection assay system, where the effect of overexpression of single candidate GTPases (Rho, Rac and Cdc42) was assessed by measuring changes in the level of GH release. Since the amounts of each candidate protein present in the cells were unknown (and not easily assessed), it may be that the levels of Cdc42 present were insufficient to stimulate secretion, especially as the data presented here indicate that regulation by Cdc42 (in mast cells at least) may be very complex. Firstly, it may involve at least two effector proteins, and secondly, there may be some cooperativity between Cdc42 and Rac in the regulation of secretion (this is discussed later). However, it seems more likely that the apparent differences in requirement for GTPases simply reflect differences between the two types of secretory cell, since cells of endocrine origin such as PC12 cells, unlike mast cells, have no absolute requirement for guanine nucleotides in the stimulation of secretion, although they can exert modulatory effects (Bittner et al., 1986; Morgan and Burgoyne, 1990b; Burgoyne et al., 1989; Bader et al., 1989; Knight and Baker, 1985) (see Introduction for details).

It is reasonable to think that preactivated rCdc42Hs interacts directly with its effectors to induce secretion, bypassing the need for exchange factors to catalyse protein activation. The existence of a Ca\(^{2+}\)-dependent exchange factor for GTP-binding proteins in mast cells has been suggested previously (Lillie and Gomperts, 1992b) and in the yeast Saccharomyces cerevisiae, there is a Ca\(^{2+}\)-binding protein, Cdc24 (Ohya et al., 1986; Miyamoto et al., 1987), which exhibits guanine nucleotide exchange factor activity for Cdc42Sc (Zheng et al.,
There is evidence that mutations within the region thought to mediate nucleotide exchange confer a Ca\(^{2+}\)-sensitive phenotype, suggesting that Ca\(^{2+}\) may be involved in exchange activity (Miyamoto et al., 1991). However, Cdc24 appears to be able, at least \textit{in vitro}, to catalyse nucleotide exchange on Cdc42Sc in the absence of added Ca\(^{2+}\) (Zheng et al., 1994b), though this may not reflect the physiological situation.

It is interesting to speculate that a similar Ca\(^{2+}\) sensitive exchange factor exists for Cdc42 in mast cells, but due to time limitations, I was unable to investigate this. One would need to measure the effect of non-activated Cdc42 on mast cell secretion in response to the dual stimulus, (and also the Ca\(^{2+}\) dependence of any activity detected). Even then, it seems likely that any results obtained may have proved inconclusive, since the recombinant GTPases cannot interact with the exchange factor smg GDS (Shirataki et al., 1991; Mizuno et al., 1991; Hori et al., 1991), and it is possible that the exchange factors present in mast cells might exhibit the same selectivity. Hence, an alternative source of protein (i.e. post-translationally modified) would be necessary for use in any further investigations carried out using rundown assays similar to those presented here. The dependence of secretion on the concentration of preactivated rCdc42Hs (Figure 5.2a) reveals that, as with Rac, the presence of GTP\_S causes an extra increment of secretion over that which occurs in response to Ca\(^{2+}\) alone. This finding is not wholly unexpected, since the added nucleotide would be expected to activate any native Rac still remaining in the cells ((Brown et al., 1997) and Chapter 4). Of course, it is also possible (indeed highly likely) that other GTP-binding proteins such as Rho (Price et al., 1995), and the heterotrimeric G\(_3\) (Aridor et al., 1993) are also activated under these conditions. These concentration-effect relationships do, however, reveal two very striking responses of the cells to preactivated rCdc42Hs which may be significant.

Firstly, the presence of GTP\_S in the dual stimulus causes a 6 fold enhancement in the sensitivity to rCdc42Hs (Figure 5.2a) and this implies the involvement of further GTP-binding proteins. The nature of the mechanism can only be speculated upon though there are a few possibilities that can be considered. One is that the protein may be a heterotrimeric G-protein, in particular G\(_{13}\), but the
substitution of [AlF₄]⁻ for GTPγS in an experiment similar to that shown in Figure 5.2a had no effect on the sensitivity to preactivated rCdc42Hs (A. O'Sullivan, unpublished observation). A more obvious candidate could be Rac, but as already shown (Figure 5.6), the presence of Rac also failed to induce a shift in sensitivity to the rCdc42Hs. (This experiment did, however, provide other important clues regarding the regulation of mast cell secretion by Rac and Cdc42, and these are discussed later).

Another candidate is Rho, previously shown to have an activating effect on mast cell secretion (Price et al., 1995; Komuro et al., 1996b). However, a recent investigation carried out on RBL (rat basophilic leukaemia) cells has revealed that selective inactivation of Rho is without effect on secretion (Prepens et al., 1996) implying that it is not an essential component of the regulatory mechanism. It is, however, widely accepted that both Rho and Cdc42 are regulators of focal adhesion assembly, and evidence of cross-talk between these two proteins in their regulation of this process has come with the finding of a sequential activation of Cdc42 followed in turn by activation of Rac and then Rho (Nobes and Hall, 1995), and also in the identification of a specific GAP for Rho and Cdc42 (but not Rac) which associates with FAK (Focal Adhesion Kinase) (Hildebrand et al., 1996). A Cdc42 GAP has also been isolated from platelets which exhibits weak GAP activity (relative to Cdc42 GAP activity) on Rho (Hart et al., 1991). Also, there is evidence for the coupling of Rho and Cdc42 signalling pathways in S. Cerevisiae (Bender et al., 1996). Cells expressing mutant forms of Boi1p, a Cdc42Sc binding protein, exhibited growth defects which could be suppressed by overexpression of Rho3p. This in turn implies that Boi1p (and also the closely related protein Boi2p) may play a role in the activation and/or function of Rho3p in this organism.

Yet another candidate GTP-binding protein which may impinge on the Cdc42 signalling pathways is Ral, another ras-related small GTPase. Ral binding proteins have been identified which exhibit GAP activity towards Cdc42 (Cantor et al., 1995; Jullien Flores et al., 1995; Park and Weinberg, 1995). There is also some evidence for the involvement of Ras in Cdc42 signalling, largely due to the demonstration of an interaction between RasGAP and p190 RhoGAP (which is also active on Cdc42 (Settleman et al., 1992; McGlade et al., 1993; Schmid et al., 1996)), although the significance of this remains uncertain. However, there is
evidence for direct interplay between these two GTP-binding proteins in the
demonstration that in *S. Pombe*, scd2 (homologous to BEM1 in *S. cerevisiae*,
required for normal cell shape and conjugation), and ras1 converge upon scd1
(homologous to Cdc24 in *S. cerevisiae*), which then interacts with Cdc42Sp in the
regulation of cell morphology and mating. In addition, it appears that these four
proteins can act cooperatively to form a complex (Chang et al., 1994). Others have
reported that Cdc42Sc can act downstream of RAS2 (on the same pathway), in the
regulation of filamentous growth in *S. cerevisiae* (Mosch et al., 1996). However,
these are long-term interactions requiring hours and secretion occurs in seconds.
While Ras and Cdc42 may cooperate in some in some aspects of mast cell
function it does not necessarily follow that they do so in the acute regulation of
secretion. This is supported by the finding that micro-injection of oncogenic Ras
protein into mast cells only induced degranulation after a number of hours (Bar-
Sagi and Gomperts, 1988). Although this cannot be directly compared to my data
due to the difference in experimental approach, it seems unlikely that Ras is
involved in the rapid responses regulated by Cdc42 under discussion here.

To summarise, it appears that a GTP-binding protein is capable of enhancing the
sensitivity of the mast cell secretory response to preactivated rCdc42Hs (Figure
5.2a) but no there are no clues as to its identity. There are many examples of
GTP-binding proteins which impinge upon Cdc42 signalling pathways in the
regulation of other processes but these may have little relevance to the regulation
of an acute process such as exocytosis.

The second significant observation was that the range of concentrations over
which rCdc42Hs exerts a stimulatory effect is more extended than that of
preactivated Rac (Figure 5.2, 5.3, compare Figure 4.11). This implies that the two
GTPases interact with the downstream pathways leading to exocytosis in a
different manner, with rCdc42Hs being capable of activating two effectors, only
one of which interacts with rRac2. Candidates to be considered for the shared
effector must include the “CRIB” (Cdc42/Rac interactive binding) containing
proteins (Burbelo et al., 1995) (such as α, β, and γ PAK (Manser et al., 1994;
Manser et al., 1995; Teo et al., 1995; Prigmore et al., 1995)), PI-3kinase (Tolias et
al., 1995; Zheng et al., 1994a) and the related proteins IQGAP1 and
IQGAP2 (Hart et al., 1996; Brill et al., 1996). However, the IQGAPs show signs
of actin co-localisation or binding and have been postulated to exert their effects via cytoskeletal changes, now thought to be dispensable in the control of exocytosis from mast cells (Norman et al., 1996).

It seems appropriate to include GAPs in the list of possible effectors which can interact with both Rac and Cdc42 since there is some evidence to suggest that they may also fulfil the role of effectors in addition to regulating GTPase activity. For example, n-chimaerin, which has GAP activity, acts synergistically with Rac and Cdc42 in the induction of morphological changes without enhancement of GTPase activity (Kozma et al., 1996). Other examples of GAPs shown to be active on both Rac and Cdc42 include some Ral binding proteins (Jullien Flores et al., 1995; Park and Weinberg, 1995), ABR (Tan et al., 1993), and RhoGAP (Lancaster et al., 1994).

It is not too surprising to find evidence of an effector in the regulation of secretion which is accessed only by Cdc42 (at least not by Rac). While there are examples of proteins which bind to Cdc42 but not to Rac, I am not aware of any which bind Rac but not Cdc42. These must be considered as candidates for the “Cdc42 unique” effector involved in secretory regulation in mast cells. Both the tyrosine kinase ACK (activated Cdc42-associated kinase) (Manser et al., 1993) and also the WAS (Wiskott-Aldrich syndrome) protein WASp (Symons et al., 1996) appear to interact specifically with Cdc42 (though there may also be a weak interaction of WASp with Rac1 (Kolluri et al., 1996)). Defects in the WAS protein appear to be responsible for the abnormalities of Wiskott Aldrich syndrome, which are limited to cells of haematopoietic lineage. Hence it may be that this protein is an effector for Cdc42 in mast cells, but again, like the IQGAPs already mentioned, this protein is postulated to exert its effects via the cytoskeleton (Symons et al., 1996; Kolluri et al., 1996) and so may not be involved in the regulation of secretion.

Two GAPs have also been identified which interact preferentially with Cdc42. These are the 25kDa platelet Cdc42 GAP (Hart et al., 1991; Barfod et al., 1993) and the Ral binding protein RalBP1 which has GAP activity towards Cdc42 (Cantor et al., 1995; Jullien Flores et al., 1995). As already suggested these may act as effectors as well as GAPs.
The inhibition of secretion by rT17N—Cdc42Hs (Figure 5.8) supports the idea that Cdc42 is a physiological regulator of secretion. It implies the existence in mast cells of an exchange factor capable of interacting with (and presumably catalysing nucleotide exchange on) Cdc42 (Farnsworth and Feig, 1991; Quilliam et al., 1994), which participates in the regulation of secretion. In addition, the leakage of Cdc42 from mast cells (Figure 5.9) following permeabilisation supports the idea that exogenous preactivated rCdc42Hs exerts its stimulatory effect on secretion by replacing the endogenous protein that has leaked from the cells.

It has been proposed that in the yeast *S. cerevisiae*, Cdc42Sc lies downstream of a heterotrimeric G-protein, and upstream of Ste20 (yeast homologue of PAK) (Simon et al., 1995; Zhao et al., 1995). Is it possible that a similar signalling sequence is involved in the regulation of mast cell secretion, with Cdc42 lying on the pertussis toxin sensitive pathway stimulated by receptor mimetic agents such as mastoparan and compound 48/80? Another possibility is that Cdc42 marks the point of divergence of two signalling pathways. As suggested by my results, Cdc42 interacts with two effectors in the regulation of secretion, and so, in the absence of Rac activation, it may be capable of activating two separate downstream pathways in response to activation of a single upstream pathway. It is also possible that Rac and Cdc42 can activate the same effector under certain conditions, but each does so in response to a different physiological stimulus. Thus Rac may lie on the pertussis toxin insensitive pathway activated by specific antigens (and Ca^{2+} ionophores), and Cdc42 may lie on the pertussis toxin sensitive pathway, but each may activate a common downstream pathway leading to secretion via the common effector. Clearly, Cdc42 is capable of regulating two pathways *in vitro*, and there is probably a complex interrelationship or cooperativity between the two GTPases in their roles as regulators of secretion. More work is needed to elucidate the mechanisms of action of both proteins, and I believe that identification of the effectors involved would constitute a significant step forward.
CHAPTER 6

"FINAL DISCUSSION AND FUTURE WORK"
FINAL DISCUSSION AND FUTURE WORK

This thesis describes the identification of a GTP-binding protein which may be involved in the regulation of exocytosis. Rac1, purified from bovine brain as a complex with RhoGDI retards the loss of responsiveness to stimulation by Ca\(^{2+}\) and GTP\(\gamma\)S (rundown) of permeabilised mast cells. In contrast RhoGDI, applied alone under the same conditions accelerates the rundown and this suggests that Rho family proteins other than Rac may be involved in regulating mast cell secretion.

The case for Rac is supported by the finding that recombinant Rac2, expressed and purified from *E. Coli*, can also retard the rundown of mast cells stimulated by the dual effectors, Ca\(^{2+}\) plus GTP\(\gamma\)S. In addition, cells treated with this protein exhibit GTP-\(\gamma\)-S-independent secretion. However, the recombinant protein is only capable of producing these effects when "pre-activated" by GTP\(\gamma\)S binding. This probably reflects the lack of post-translational modification known to be required for translocation to the plasma membrane (Abo et al., 1994; el Benna et al., 1994; Quinn et al., 1993; Didsbury et al., 1990; Heyworth et al., 1994) and interaction with some exchange factors (GEFs) which catalyse activation (Ando et al., 1992; Heyworth et al., 1993; Takai et al., 1993; Bokoch et al., 1994; Shirataki et al., 1991; Mizuno et al., 1991; Hori et al., 1991). The dominant inhibitory mutant rT17N-Rac2 inhibits secretion induced by Ca\(^{2+}\) and GTP\(\gamma\)S, and Rac has been shown to leak from the permeabilised mast cells under the conditions in which they lose responsiveness (Brown et al., 1997), lending further credence to the idea that Rac is an endogenous regulator of secretion. However, the presence of free GTP\(\gamma\)S in addition to the preactivated rRac2 causes an enhancement of the secretory response and this must implicate at least one other GTP-binding protein in the regulatory mechanism. This may not be a member of the Rho family, since an optimum concentration of RhoGDI fails to inhibit mast cell secretion completely (Mariot et al., 1996).

One candidate must be Cdc42, since preactivated recombinant Cdc42Hs can also retard the rate of rundown of the secretory response and, like rRac2, it is also capable of eliciting GTP\(\gamma\)S-independent secretion. The dominant inhibitory
mutant rT17N-Cdc42Hs inhibits secretion induced by Ca\(^{2+}\) and GTP\(\gamma\)S and with Cdc42 leaking from SL-O permeabilised mast cells it appears that this is another GTPase regulator of secretion. Interestingly, the dose-response relationships for rRac2 and rCdc42Hs in the stimulation of secretion from run-down mast cells stimulated with either Ca\(^{2+}\) alone or by the dual effector system are different. rCdc42Hs characteristically stimulates secretion when applied over a much wider range of concentrations than rRac2.

When present at low concentrations rCdc42Hs enhances the extent of secretion due to the presence of a saturating concentration of rRac2. I have concluded that while both GTPases can interact (under these conditions) with a common effector to induce secretion, rCdc42Hs can additionally interact with a second downstream effector which is not accessible to rRac2.

If Rac and Cdc42 are indeed the authentic regulators of exocytosis (G\(E\)) in mast cells, then this may provide a rationale for a number of earlier observations. In particular, it may explain why the onset of exocytosis from permeabilised cells is delayed by the presence of ATP (but not by AppNHp) (Tatham and Gomperts, 1989). This was originally taken as an indication of a dephosphorylation step necessary for the induction of exocytosis (see Introduction - "ATP"). It has already been mentioned that the complexes of Rho-GTPases with RhoGDI are more stable when the GDI is phosphorylated (see p ??), so it is possible that dephosphorylation of RhoGDI (which would allow Rac and/or Cdc42 to dissociate and become available for activation) constitutes a required step in the activation of secretion. Dephosphorylation of RhoGDI could also explain why the rapid loss of sensitivity to stimulation in cells deprived of ATP can be restored by readdition of ATP (Howell et al., 1989), and that after this, secretion can be stimulated by Ca\(^{2+}\) alone (Churcher et al., 1990b). In the absence of ATP, the complexes may be sufficiently destabilised that the Rac and/or Cdc42 dissociate, bind to the plasma membrane and can thus activate secretion even in the absence of a guanine nucleotide. This of course, assumes that the proposal of an equilibrium state (presented in Chapter 3 "Discussion") holds, and further that the critical condition for stimulation is membrane binding, not the identity of the bound guanine nucleotide. One must assume, though, that in the absence of a stimulating guanine nucleotide the Rho proteins would be functioning less efficiently than normal, and
I would certainly expect that the stimulation of secretion by the Ca\(^{2+}\)-only stimulus is not simply due to Rac and/or Cdc42, but reflects the activity of more than one component. In particular, the requirement for ATP in the stimulation of secretion under these conditions indicates that there is another ATP-dependent step in the stimulation of secretion, the nature of which is unknown at present. However, Rac/Cdc42 activation may be rate-limiting. When mast cells are treated with okadaic acid (phosphatase inhibitor) or PMA before permeabilisation in the absence of ATP and then restored by “add-back” of ATP an activating guanine nucleotide becomes an absolute requirement once again (Howell et al., 1989; Churcher et al., 1990b). This is consistent with my proposal since it would be expected that whilst RhoGDI remains in a phosphorylated condition forming stable complexes with Rac and Cdc42, activation of secretion can only occur in the conventional manner, requiring the presence of an activating guanine nucleotide. However, as already mentioned, whilst the activation and deactivation of Rac/Cdc42 may provide a logical explanation for these observations, there is as yet no evidence for the involvement of a phosphatase in these events, and it is also unlikely that the observations are due solely to Rac/Cdc42. Other, as yet unknown mechanisms may occur alongside Rac and Cdc42 activation in the stimulation of secretion from mast cells. Indeed, this seems likely, since Rac/Cdc42 activation due to dephosphorylation of RhoGDI does not provide an explanation for the finding that ATP induces onset delays even in the absence of Mg\(^{2+}\), which itself implies that a dephosphorylation does not constitute an essential step in the pathway leading to exocytosis in mast cells (Lillie et al., 1991).

At present, there are no clues as to the mechanisms whereby Rac and Cdc42 exert their stimulatory effects on secretion. As already mentioned (Chapters 4 and 5), candidates for the common effector for these GTPases include the CRIB-containing proteins (Burbelo et al., 1995), such as PAK (Manser et al., 1994; Manser et al., 1995; Teo et al., 1995; Prigmore et al., 1995), PI-3kinase (Tolias et al., 1995; Zheng et al., 1994a), IQGAP1 and IQGAP2 (Hart et al., 1996; Brill et al., 1996), whilst candidates for the Cdc42-only effector include ACK (Manser et al., 1993) and WASp (Symons et al., 1996). So far, of course, there is no evidence for the involvement of any of these proteins in the regulation of secretion from any cell type. As already mentioned, there is evidence for the involvement of PIP-5kinase in the regulation of secretion (Hay et al., 1995), and
this protein has been shown to interact with Rac (Tolias et al., 1995). Cdc42 interacts only weakly with this enzyme (Tolias et al., 1995) making it an unlikely candidate for the shared effector. However, it may be that when high concentrations of activated rCdc42Hs are provided in the absence of activated rRac2 during the rundown assay, the rCdc42Hs will stimulate PIP-5kinase in addition to its specific effector sufficiently to induce secretion (Figure 5.2a). Even if this is the case, it is unlikely that Cdc42 interacts with PIP-5kinase in vivo, and if so, there would be no physiological “shared effector” so that under normal conditions, Cdc42 and Rac would each have their own effectors (assuming that the Rac effector is indeed PIP-5kinase).

It is clear that the next major step forward must be to identify the downstream effectors of these GTPases in the regulation of exocytosis. This could be achieved by either affinity chromatography, using activated rRac2 or rCdc42Hs as the ligand, or by a yeast two hybrid method. In addition, because a Ca$^{2+}$-binding exchange factor for Cdc42, Cdc24, has been identified in yeast (Ohya et al., 1986; Miyamoto et al., 1987; Zheng et al., 1994b), it will be interesting to investigate the Ca$^{2+}$-dependence of the secretory response to Cdc42 in mast cells (as outlined in the discussion to Chapter 5) since a similar factor may be responsible, at least in part, for the Ca$^{2+}$-dependence of secretion. However, post-translational modification is required for interaction of Rho proteins with some exchange factors (Ando et al., 1992; Heyworth et al., 1993; Takai et al., 1993; Bokoch et al., 1994; Shirataki et al., 1991; Mizuno et al., 1991; Hori et al., 1991), so a source of modified protein would be required.

My work has unearthed two possible candidate GTPases, Rac and Cdc42, for Ge, the GTP-binding protein that mediates the exocytotic process in mast cells. Since these two proteins, which until recently were never even vaguely perceived to be involved, are turning out to be regulators of many diverse cellular processes, this begs the question: are these proteins situated well upstream of the exocytotic fusion machinery? In addition, are there really, as implied by the shift in sensitivity to rCdc42Hs in the presence of GTPγS (Figure 5.2a) which was found not to be due to Rac2 (Figure 5.6), further GTPases involved? If so, are they directly involved in the fusion process, as hypothesised for Rab3? As previously discussed (Chapter 5, “Discussion”), candidate regulators of mast cell secretion
which may mediate the enhanced sensitivity to rCdc42Hs include Rho (Price et al., 1995), G_{i3} (Aridor et al., 1993) and βγ subunits (Pinxteren et al., 1997).

However, it appears that there is more than one, maybe more than two, GTPases involved in the regulation of secretion, at least in mast cells, and it remains to be seen exactly which these are, and how they carry out their duties. The GTPases continue to surprise us by their versatility, and I am sure that the surprises will not cease now. As more data become available, so the signalling pathways regulated by these proteins become more and more complex. Clearly, for those interested in dissecting these pathways, a mammoth task lies ahead.


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Purification and Identification of FOAD-II, a Cytosolic Protein that Regulates Secretion in Streptolysin-O Permeabilized Mast Cells, as a Rac/RhoGDI Complex

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Mast cells permeabilized by treatment with streptolysin-O in the presence of Ca$^{2+}$ and GTP-γ-S can secrete almost 100% of their contained N-acetyl-β-D-glucosaminidase. If these stimuli are provided to the permeabilized cells after a delay, the response is diminished and the ability of the cells to undergo secretion runs down progressively over a period of about 30 min. This is thought to be due to the loss of key proteins involved in the exocytotic mechanism. Using this effect as the basis of a biological assay, we have isolated a protein from bovine brain cytosol that retards the loss of responsiveness to stimulation by Ca$^{2+}$ and GTP-γ-S. Purification of this protein and peptide sequencing have enabled us to identify it as the small GTP-binding protein rac complexed to the guanine nucleotide exchange inhibitor rhoGDI. Both proteins are required to retard the loss of the secretory response, while purified rhoGDI applied alone accelerates the rundown.

INTRODUCTION

Although it is widely accepted that the mechanisms underlying the membrane fusion events of the secretory pathway are likely to be highly conserved, the control processes that determine if and when two membranes are to undergo fusion appear to be widely divergent. The process of regulated exocytosis, in which the membranes of secretory granules undergo fusion with the cytosolic face of the plasma membrane, provides some excellent opportunities to investigate these mechanisms and their control. Development of techniques of selective plasma membrane permeabilization that permit the direct manipulation of the composition of the cytosol in secretion-competent cells has allowed the identification of low molecular weight solutes that regulate exocytosis (Lindau and Gomperts, 1991) and these have been found to vary among different classes of secretory cells. For exocytosis of catecholamines from adrenal chromaffin cells, calcium alone is believed to be the sufficient trigger (Knight and Baker, 1982), whereas in other systems, such as the acinar cells of the parotid, secretion is thought to be dependent on a rise in the concentration of cyclic AMP (Dormer and Ashcroft, 1974; McMillian et al., 1988). For non-neuroendocrine cells such as the granulocytic mast cells (Howell et al., 1987; Lillie and Gomperts, 1992a), eosinophils (Nüssle et al., 1990; Cromwell et al., 1991), and neutrophils (Barrowman et al., 1986; Cockcroft, 1991) it appears that the final stimulus to exocytosis is provided by GTP and mediated through GTP-binding proteins.

After permeabilization all secretory cells appear to undergo a decline in responsiveness with time to stimuli (such as Ca$^{2+}$ and guanosine 5'-O-(3-thiotriphosphate) [GTP-γ-S]). This is understood to be due, at

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least in part, to the loss of cytosolic proteins (Howell and Gomperts, 1987; Srafian et al., 1987; Koffler and Gomperts, 1989). Using brain cytosols as a source, a number of proteins have been identified that can retard this loss of responsiveness. In particular, this approach has been applied to neuroendocrine cells for which an elevation of cytosol Ca\(^{2+}\) is the primary stimulus to secretion. Several regulatory proteins have been identified that can impede the onset of a refractory state. These include the lipid-dependent Ca\(^{2+}\)-binding protein annexin-II (Ali and Burgoyne, 1990), members of the 14–3-3 family (Morgan and Burgoyne, 1992; Roth et al., 1993), the catalytic subunit of CaMP-dependent protein kinase (Morgan et al., 1993), 140-kDa calcium-activated protein in secretion (Nishizaki et al., 1992), phosphatidylinositol transfer protein (PI-TP) (Hay and Martin, 1993), phosphatidylinositol-5-kinase (Hay et al., 1995), and calmodulin (Matsuda et al., 1994). We have now extended this approach to an investigation of mast cells in the hope that we could identify specific regulatory components of an exocytotic mechanism in which the stimulus is provided by activation of GTP-binding proteins.

**MATERIALS AND METHODS**

Deep-frozen bovine brains were obtained from Advanced Protein Products (Brierley Hill, West Midlands, UK). GTP-γ-S was purchased as a 100 mM stock solution from Boehringer Mannheim (Mannheim, Germany) and all other chemicals used were of the highest quality from standard commercial sources. All chromatographic columns used were obtained from Pharmacia (Uppsala, Sweden). The BCA assay for protein was carried out using a kit purchased from Pierce (Chester, UK). Streptolysin-O (SL-O) was obtained from Murex Diagnostics (Dartford, Kent, UK). Rabbit polyclonal antibodies to rac1 and 2 were purchased from Santa Cruz Biotechnology, Santa Cruz, CA. Antibody to rhoCDI was a gift from Alan Hall.

Rat peritoneal mast cells were prepared by peritoneal lavage of male Sprague Dawley rats (300–400 g) and purified to greater than 99% purity by a Percoll step gradient as previously described (Tatham and Gomperts, 1990) and treated with metabolic inhibitors (2-deoxyglucose, 2mM, and entomycin A, 10mM).

**Secretion Experiments**

The cells were permeabilized by SL-O, a bacterial cytolysin. This was applied to mast cells maintained at ice temperature at a concentration of 1.6 IU ml\(^{-1}\). After 5 min the cells were diluted into buffer A (137 mM NaCl, 2.7 mM KCl, 1 mM MgCl\(_2\), 0.02% NaN\(_3\), 20 mM piperezine-N,N,N'-triethanesulfonic acid) (Pipes), pH 6.8 supplemented with 0.1% bovine serum albumin (BSA), and the cells were washed free of unbound SL-O by centrifugation and resuspension at 4°C. To open the plasma membrane lesions and initiate rundown, 20-μl samples were added to 20 μl of run-down buffer at 37°C (buffer A plus 0.6 mM EGTA (Ca\(^{2+}\) to < pCa8), 200 mM ATP, 3 mM creatine phosphate, creatine kinase 0.6 mg ml\(^{-1}\)), and 20 μl of protein under test made up in buffer B (buffer A plus 100 μM ATP, 0.3 mM EGTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 1 mg ml\(^{-1}\) pepstatin, 1 mg ml\(^{-1}\) leupeptin). Experience indicates that secretion in response to a maximal stimulus declines after 10–20 min to approximately 10% of its normal level (these cells are capable of releasing 100% of their contained secretory materials). The cells were then stimulated to secrete by addition of 20 μl stimulation (or control) buffer (buffer A containing GTP-γ-S [zero or 200 μM] plus 3 mM EGTA with CaCl\(_2\) to regulate the level of free Ca\(^{2+}\) at pCa7 or pCa5) estimated using the program Ligandy; Gomperts et al., 1992). After a further 20-min incubation, the release reaction was quenched by the addition of 100 μl ice cold buffer A. The cells were sedimented and soluble hexosaminidase was assayed as previously described (Gomperts et al., 1992). Secretion is expressed as the % of total cellular N-acetylβ-glucosaminidase (hexosaminidase) released by 100 μM GTP-γ-S and 10 μM Ca\(^{2+}\) (pCa5), above that released by 100 mM Ca\(^{2+}\) (pCa7). All determinations were carried out in quadruplicate unless otherwise stated.

For analysis of activity in the reconstitution of secretion, column fractions were exchanged into buffer B before assay by the use of NAP-5 buffer exchange columns.

**Protein Purification**

Deep-frozen bovine brains were stored at —80°C. Generally we have used two brains for each preparation. These were defrosted at 4°C for 8 h and then homogenized in buffer B (1 liter per 500 g) using a Waring blender. The homogenate was centrifuged at 14,400 × g overnight at 4°C and the supernatant was then subjected to fractionation by the procedures described below.

The supernatant was initially fractionated by ammonium sulphate precipitation. Material capable of reconstituting the activity of cells rendered partially refractory by prior permeabilization was found to be present in proteins precipitating at 85% but remaining soluble at 60% of saturation. The cytosol was stirred for 1 h at 4°C with 60% saturated (NH\(_4\))\(_2\)SO\(_4\) and then centrifuged at 14,400 × g for 30 min. The pellet was discarded and the precipitation was repeated at 85% saturated (NH\(_4\))\(_2\)SO\(_4\). After centrifugation, the resultant pellet was resuspended in 50 μl buffer B and applied to an octyl sepharose column (120 ml, XK50 column) equilibrated in 2 M (NH\(_4\))\(_2\)SO\(_4\), 20 mM Pipes, pH 6.5.

All buffers used for chromatography contained 0.02% NaN\(_3\) to prevent microbial growth and all chromatography steps were carried out on a fast performance liquid chromatography at 4°C to reduce proteolysis. Proteins were eluted from the octyl sepharose column using a linear gradient down to 20 mM Pipes, pH 6.5, with a flow rate of 5 ml min\(^{-1}\). The active fractions were pooled and analyzed overnight against 20 mM Tris-HCl, pH 8.5. This was then applied to a Q-sepharose-FF column, (60 ml, XK26 column) pre-equilibrated with 20 mM Tris-HCl, pH 8.5, and proteins were eluted with an increasing gradient of NaCl with a flow rate of 5 ml min\(^{-1}\). The active fractions were then concentrated and then applied to a 5 ml Hi-Trap SP column and eluted with a gradient of NaCl at a flow rate of 2 ml min\(^{-1}\). At this stage two activities were recovered. The active fractions from the second peak were pooled and concentrated to 1 ml on a Centricon (Amicon, Beverly, MA) spin filter having a 10 kDa cut-off. This was injected on to a G75 preparation grade gel filtration column (Hi Load 16/60 Superdex 75pg) pre-equilibrated in buffer A (flow rate 0.12 ml min\(^{-1}\)), and the activity eluted as a single peak with an estimated molecular weight of 43 kDa. The active fractions were then again pooled and exchanged into 20 mM Tris-HCl, pH 8.5, and then applied to a monoQ column (1 ml) from which a stimulatory activity and an inhibitory activity were recovered. The stimulatory activity was then rechromatographed on the G75 column.

Protein concentration was assayed by the BCA method. The peptide composition of column fractions was analyzed on 12% SDS polyacrylamide gels using the method of Laemmli (1970) and visualized by silver staining (Morrissey, 1981).

Protein sequencing samples were pyridylethylated before analysis according to a slight modification of a published method (Thomson and Bayne, 1988). Briefly, the sample was concentrated to dryness on an Amicon 3 (3-kDa cut-off; Beverly, MA) spin filter and resuspended in 70 μl guanidinium buffer (6 M guanidinium HCl, 0.25 M
Typically these cells can respond to the stimulus by membrane lesions), or at various times thereafter. which allows the pre-bound SL-O to generate plasma by application of a stimulus (pCa5 with 100 μM GTP-

Antibody binding was detected using the Amersham ECL kit (Buckinghamshire, UK) according to instructions. Preliminary work was carried out with extracts of fresh rat brain. When rat brain cytosol proteins at 2 mg ml⁻¹ are provided to permeabilized mast cells during the run-down period before stimulation, the rate of decline of the secretory responsiveness to stimulation by 10 μM Ca²⁺ with 100 μM GTP-γ-S is reduced. This activity was found to be sensitive to heat and to treatment with trypsin. Initial fractionation of the rat brain cytosol by (NH₄)₂SO₄ precipitation indicated the activity to be present in the fraction sedimenting at 60–85% of saturation. Although crude bovine brain cytosol was found to be ineffective, the fraction sedimented by (NH₄)₂SO₄ at 60–85% saturation was again

Leakage of RhoGD1

Cells were purified and treated with 100 μM diisopropyl phosphofluoridate for 10 min and then with metabolic inhibitors as described above. They were then permeabilized as usual and samples (0.3 × 10⁶) were taken at various times of rundown. Cells were immediately sedimented by centrifugation and the proteins present in the supernatant were aggregated by the addition of ice-cold acetone and precipitated by centrifugation. Precipitated proteins were then dissolved in sample buffer and run on a 12% polyacrylamide gel. Proteins were transferred to nitrocellulose which was probed with an antibody (anti-rhoCD1 from Transduction Laboratories, Lexington, KY) according to the manufacturer's instructions. Antibody binding was detected using the Amersham ECL kit (Buckinghamshire, UK) according to instructions.

All data reported are representative of experiments that were repeated on at least three separate occasions. The purification shown in Figures 1–6 derives from a single preparation that was repeated on at least 25 occasions.

RESULTS

This paper describes the isolation of a factor that modulates secretion (degranulation) from permeabilized mast cells by altering the rate of onset of a refractory state understood to be due to leakage of cytosol proteins. Figure 1 illustrates the extent of secretion elicited by application of a stimulus (pCa5 with 100 μM GTP-γ-S) to mast cells either at the time of permeabilization (i.e. the time of elevation of the temperature to 37°C, which allows the pre-bound SL-O to generate plasma membrane lesions), or at various times thereafter. Typically these cells can respond to the stimulus by releasing close to 100% of their contained hexosaminidase, but after a period of about 10 min this declines to about 50% and then to zero at about 30 min (nb the actual timecourse of rundown varies widely between experiments). As shown in Figure 1, the factor we have isolated retards the rate of decline; we have called this activity FOAD-II, a factor of activation of degranulation.

Preliminary work was carried out with extracts of fresh rat brain. When rat brain cytosol proteins at 2 mg ml⁻¹ are provided to permeabilized mast cells during the run-down period before stimulation, the rate of decline of the secretory responsiveness to stimulation by 10 μM Ca²⁺ with 100 μM GTP-γ-S is reduced. This activity was found to be sensitive to heat and to treatment with trypsin. Initial fractionation of the rat brain cytosol by (NH₄)₂SO₄ precipitation indicated the activity to be present in the fraction sedimenting at 60–85% of saturation. Although crude bovine brain cytosol was found to be ineffective, the fraction sedimented by (NH₄)₂SO₄ at 60–85% saturation was again
found to contain FOAD activity. This material comprised the starting point for further fractionation. We have no reason to believe that other activities might not be present in proteins sedimenting at lower concentrations of (NH₄)₂SO₄ but the advantage of the high salt fraction for our work at this stage was that it has low levels of contaminating hexosaminidase activity. Because of this, the bioassay procedure, involving the measurement of hexosaminidase secretion, was much easier.

The 60–85% pellet was resuspended in buffer B, made up to 2 M (NH₄)₂SO₄ by addition of an appropriate volume of 3.4 M concentrated stock and applied to an octyl sepharose column (Figure 2), which was used to fractionate the proteins according to their hydrophobicity. Unadsorbed proteins (i.e., the most polar proteins) were first washed through with 170 ml of 2 M (NH₄)₂SO₄ (fractions 1–17) and the column was then eluted with a declining salt gradient down to 0.5 M (NH₄)₂SO₄ (fractions 18–79). Fractions 45–63, eluting at 1.25–0.83 M (NH₄)₂SO₄ were found to contain FOAD activity. These were pooled and dialyzed overnight at 4° against 2 M (NH₄)₂SO₄ and then diluted to 2 M (NH₄)₂SO₄ by addition of an appropriate volume of 3.4 M concentrated stock and applied to a Q-Sepharose column (anion exchange)-fast flow column (Figure 3) that was eluted with a linear gradient of NaCl. FOAD activity was found to elute at 0.096–0.128 M NaCl. An inhibitory activity that emerged when the salt concentration was taken above 0.2 M was discarded. The active material (fractions 22–27) were pooled and concentrated under pressure to 3 ml using an Amicon YM-10 filter. The sample was then applied to three NAP-10 columns to exchange the buffers into 20 mM Pipes, pH 6.9, and applied to a Hi-Trap SP (cation exchange) column that was eluted with a gradient of NaCl.

The SP column resolves the FOAD activity into two components (Figure 4). The first of these to emerge, FOAD-I, does not bind to the column and elutes in the flow-through volume, before the NaCl gradient is applied. FOAD-II elutes at 80–220 mM NaCl (fractions 17–26). We have concentrated on the FOAD-II component and the FOAD-I has not so far been further purified.

The fractions containing FOAD-II were concentrated to 1 ml using a Filtron Microsep centrifugal concentrator (Brooklyn, Australia) having a 10-kDa cut-off filter and the material was then injected onto a G75 (gel filtration) column that was eluted with 20 mM PIPES, pH 6.8. Activity was found to elute at 58.4 ml (approximately 43 kDa) (Figure 5A). The proteins from the fractions containing active material were subjected to analysis by SDS-gel electrophoresis and were found to contain two peptides of 22 and 28 kDa (see Figure 5B). The ratio of staining of the two peptides is dependent on the technique; although on silver stain the intensity of the 22-kDa band is the greater, when visualized by colloidal Coomassie blue (Neuhoff et al., 1988), the 28-kDa band is more intense (our unpublished observations). Analysis by 32P-GTP overlay of the peptides following electrophoretic transfer on to Immobilon-P revealed that the 22-kDa component is a GTP-binding protein (our unpublished observations).

Figure 2. Chromatography of FOAD on octyl Sepharose. Brain cytosol proteins precipitated by 60–85% saturated (NH₄)₂SO₄ were dissolved in buffer B and then made up to 2 M (NH₄)₂SO₄ and loaded onto octyl Sepharose. This was eluted with a gradient (0–0.5 M (NH₄)₂SO₄ and 10-ml fractions were collected. Small samples (0.5 ml) of the fractions were passed through NAP-5 columns for exchange into buffer B, suitable for bioassay. Fractions (45–63) eluting in the range 1.2–0.8 M (NH₄)₂SO₄, which contained FOAD activity, were pooled and dialyzed overnight against 20 mM Tris-HCl, pH 8.5, for loading onto a Q-Sepharose (anion exchange) column. Solid symbols indicate protein concentration (A₅₉₀), open symbols indicate FOAD activity, and continuous line indicates concentration of salt gradient.

Figure 3. Chromatography of FOAD on Q-Sepharose. The active material eluted from chromatography on octyl Sepharose was loaded onto a Q-Sepharose column at pH 8.5 and eluted with an increasing gradient of NaCl (0–0.2 M). Ten-milliliter fractions were collected. Samples (0.5 ml) of the fractions were passed through NAP-5 columns for buffer exchange into buffer B, suitable for bioassay. Fractions (22–27), eluting in the range 0.08–0.12 M NaCl that contained FOAD activity were combined and concentrated to 3 ml on a YM10 membrane and then exchanged into 20 mM Pipes, pH 6.9, using 3 NAP-10 columns for application to a Hi-Trap SP column. Symbols are as for Figure 2.
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Figure 4. Chromatography of FOAD on Hi-Trap SP. The active material eluted by chromatography on Q-Sepharose was loaded onto a 5-ml Hi-Trap SP (cation exchange) column at pH 6.9 and eluted with an increasing gradient of NaCl (0–0.4 M) and 1-ml fractions were collected. Samples were removed, prepared, and assayed for FOAD activity. Active material was found to elute in the flow-through buffer (zero salt, nonadherent) and again in fractions 17–26 (0.06–0.24 M NaCl). The nonadherent activity was discarded and the active fractions from the second peak were pooled and concentrated to 1 ml on a Centricon spin filter having a 10-kDa cut off, in preparation for gel filtration chromatography. Symbols are as for Figure 2.

In our attempts to resolve the 43-kDa active material further, we found that any procedure that separated the 22- and 28-kDa components completely had the effect of destroying the ability of FOAD-II to retard rundown of the permeabilized cells. This indicated that both peptides are required for the activity. However, using a monoQ (anion exchange) column we found that the activity could be resolved into an activating and an inhibitory component. The column was eluted with a gradient of NaCl buffered with Tris at pH 8.5. Active material eluted at 160–200 mM NaCl and the inhibitory component emerged at 220–240 mM NaCl (Figure 6A). The resolution of these peptides is sensitive to the flow rate of the eluting solvent. At flow rates greater than 0.5 ml min⁻¹ the fine resolution of the stimulatory peak is lost and there is a concomitant increase in the inhibitory fraction. Analysis of the fractions by SDS-gel electrophoresis (Figure 6B) showed that FOAD activity again correlates with the presence of the 22- and 28-kDa peptides, although the ratio 22 kDa:28 kDa is now higher than that of the active G75 fractions, while the inhibitory component appears to consist of the 28-kDa protein alone. The active material from the monoQ anion exchange column was concentrated to 1 ml using a Filtron centrifugal concentrator and applied to a G75 column, and after analysis of the active fractions by SDS-gel electrophoresis (Figure 6C) was found to consist only of the 22-28-kDa complex. The monoQ column appears to allow partial separation of the complex of 22- and 28-kDa peptides, providing two components, one of which is the original active complex, the other, 28 kDa, causing inhibition. The monomeric 22-kDa component, which appears to be inactive, co-elutes with the complex and can be removed by gel filtration.

The purified proteins emerging from the second G75 column were subjected to sequence analysis. From the 22-kDa protein, four peptides, representing 47% of the total, were sequenced (see Table 1) and these were found to match the human ras-like protein TC25 (human rac1) with 97.8% identity (by comparison there is 92.0% identity with human rac2, with seven specific differences being consistent with rac1 rather than rac2). We have been unable to obtain any sequence for the C-terminus, and antibodies raised against C-terminal peptides from human rac1 and rac2 showed no cross-reactivity (Western blotting) with the bovine protein that we isolated, nor did they react with pro-
Figure 6. Chromatography of FOAD-II on monoQ Sepharose. (A) The 43-kDa protein eluted from a gel filtration column was loaded onto a monoQ column. This was eluted with a gradient of NaCl (0-0.4 M) and 0.5-ml fractions were collected. Samples were removed and exchanged using NAP-5 columns into buffer B for bioassay. Two activities, coinciding with the emergence of protein (Ago trace) were detected, the first of which (FOAD-II) retarded the rundown in the secretion assay, the second being inhibitory. The pooled material from the stimulatory peak was concentrated and reapplied to a G75 Sephadex column. Symbols are as for Figure 2. (B) Samples from fractions 21-28 were extracted into standard Laemmli sample buffer (Laemmli, 1970) under denaturing conditions and the proteins were separated on 12% SDS gels. Visualization was by silver staining. (C) SDS gel analysis of FOAD-II-containing fractions after re-chromatography by gel filtration on Superdex G75pg.

proteins present in bovine brain cytosol. We are therefore unable to determine conclusively whether the GTP-binding protein that we have isolated is rac1 or rac2, however, as there is apparently no mRNA coding rac2 in (human) brain (Didsbury et al., 1989) the protein is likely to be rac1. Two peptides, representing 28% of the total protein, were sequenced from the 28-kDa protein (see Table 2) and these were found to match the sequence of bovine rhoGDI. An antibody against RhoGDI reacted (Western blotting) against the 28-kDa protein (our unpublished observations).

RhoGDI leaks rapidly from the permeabilized cells and this is effectively complete within 5 min as shown by analysis of the cell-conditioned salts solution (Figure 7). Due to the nonavailability of suitable antibodies we have been unable to carry out a similar analysis for the leakage of rac.

Figure 8 illustrates the concentration-activity relationship of the rac/rhoGDI complex in the reconstitution of secretory activity in partially run-down mast cells. In this experiment the permeabilized mast cells were incubated in the presence of various concentrations of the complex under nonstimulating conditions (pCa8) and allowed to run down to the point that no more than 20% secretion could be elicited on stimulation (pCa5 with 100 μM GTP-γ-S) in the absence of added protein. The extent of induced secretion increased with increasing concentrations of FOAD. The optimal concentration was found to be in the range of 5 to 15 μg ml⁻¹ (four different preparations) with EC₅₀ of 2.6 ± 0.4 μg ml⁻¹ (i.e., 50 ± 8 nM) (mean ± SEM, n = 4 experiments, each determination carried out in triplicate). At no concentration in the range tested did the FOAD have any effect at pCa7. As shown in the experiment illustrated in Figure 1 (see above) illustrating the effect of the complex on the rate of rundown of the secretory response, it is only capable of enhancing secretion in cells that still maintain some residual response to stimulation.

Under the normal operating conditions of these experiments, secretion from mast cells requires the presence of both Ca²⁺ and an activating guanine nucleotide. The dependence on these two effectors is best presented in the form of a matrix in which the concentrations of both are treated as variables. Such an experiment is illustrated in Figure 9 in which permeabilized mast cells were first allowed to run down in the presence (or absence) of an optimum concentration of FOAD-II and then stimulated with various concentrations of GTP-γ-S and Ca²⁺. When the cells run down, both the extent of maximal secretion and the sensitivity to the two effectors declines. In the experiment illustrated, the cells were allowed to run down to the extent that the stimulus (pCa5 and 100 μM GTP-γ-S) induced about 5% secretion. Provision of FOAD enhanced this secretion in a manner that was dependent on the concentrations of both effectors. Even so, there was very little secretion unless the maximal concentrations were provided. (This result can be compared with the concentrations required to
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Table 1. Sequence analysis and matching of FOAD-II peptide with human TC25

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>Matches</th>
<th>Mismatches</th>
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<tr>
<td>P15153 Human rac2</td>
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<td></td>
<td></td>
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<tr>
<td>P15154 Human ras-like protein TC25 (human Rac1)</td>
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<td></td>
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<td>Rac2</td>
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Bold type indicates amino acids sequenced, uppercase indicates matches, and lowercase indicates mismatch with published sequence racl obtained from the Swiss-Prot Database. * indicates mismatch of sequence with rac2.

stimulate secretion from cells triggered at the time of permeabilization: in the presence of ATP and 100 μM GTP-\(\gamma\)-S, secretion can be induced at any concentration of Ca\(^{2+}\) above pCa6.5; conversely, for cells permeabilized in the presence of Ca\(^{2+}\) at pCa5, secretion commences at any concentration of GTP-\(\gamma\)-S above 10\(^{-6}\) M [Howell et al., 1987; Gomperts and Tatham, 1988]). Surprisingly, for FOAD-II-supported cells, Ca\(^{2+}\) alone (i.e., in the absence of GTP-\(\gamma\)-S) at the highest concentration (pCa5) is able to elicit a small (4%), but significant extent of secretion. It is unlikely that this secretion is due to in situ generation of GTP by transphosphorylation from ATP since deoxyUDP (1 mM), a competitive inhibitor of nucleotide diphosphate kinase, was without effect. The dependence on the concentration of FOAD-II for such Ca\(^{2+}\)-induced secretion is the same as when GTP-\(\gamma\)-S is also provided.

The separation of the FOAD-II complex into two components by chromatography on monoQ anion exchange resin (Figure 6) revealed that rhoGDI is inhibitory to exocytosis in run-down cells. The experiment illustrated in Figure 10 shows that the purified rhoGDI accelerates the rate of rundown of the permeabilized cells. It is possible that this reflects the detachment and leakage of membrane-bound components of the rho family of low molecular weight GTP-binding proteins, including Rac. The extended lag, about 5 min, before the onset of inhibition is probably due to the time required for diffusion of the exogenous rhoGDI, the solubilization of the rho and Rac proteins, and their diffusion to the exterior. (One would expect that soluble complexes of the GTP-binding proteins with the rhoGDI should be active when present inside the cells).

Table 2. Sequence analysis and matching of FOAD-II peptide with bovine rhoGDI

<table>
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<th>Peptide</th>
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<th>Mismatches</th>
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Bold type indicates amino acids sequenced, uppercase indicates matches, and lowercase indicates mismatch with published sequence of bovine rhoGDI obtained from the Swiss-Prot Database.
Figure 7. Leakage of rhoGDI from mast cells permeabilized by treatment with streptolysin-O. Cells pretreated with diisopro pylphosphofluoridate to prevent proteinolysis, were treated as detailed in the legend to Figure 1 and timed samples were removed as indicated. The cells were immediately sedimented and the proteins present in the external salts solution were separated by SDS-gel electrophoresis. After electrophoretic transfer to nitrocellulose, the proteins were probed with an antibody to rhoGDI and detected by the ECL technique. The figure illustrates relative density of each band by densitometry and the inset shows the appearance of the ECL film.

In this assay, we also tested phosphatidylinositol transfer protein (PI-TP), 14-3-3 proteins (Exo1), NSF (NEM sensitive factor), α-SNAP, γ-SNAP (soluble NSF attachment protein), calmodulin, and annexin-II, all known to modulate exocytosis in other secretory systems. These were all tested at concentrations known to support secretion in other cells. None of these proteins had any discernible effect on the rate of rundown of permeabilized mast cells (our unpublished observations). Three of these (α-SNAP, γ-SNAP, and NSF) were subsequently tested on cultured bovine adrenal chromaffin cells and shown to be active in that system (Morgan and Burgoyne, personal communication).

DISCUSSION

Mast cells, permeabilized with streptolysin-O and stimulated simultaneously with Ca^{2+} and GTP-γ-S, can undergo total degranulation and release 100% of their contained hexosaminidase and histamine. If the stimulus is provided some minutes after the cells are permeabilized, then the extent of release is reduced. This phenomenon is referred to as rundown and probably occurs, in part at least, as a consequence of the leakage of proteins through the lesions in the plasma membrane induced by SL-O. For example, leakage of lactate dehydrogenase (Howell and Gomperts, 1987) and phosphoglycerate kinase (Gomperts et al., 1987) is rapid, being essentially complete by 5 min and the competence of cells to undergo stimulated exocytosis extends well beyond this time. Although the rate of rundown varies considerably between experiments, our experience indicates that the sensitivity to stimulation extends well beyond 20 min before diminishing to zero. For this reason, the decline in responsiveness is unlikely to be related to the loss of true soluble proteins of the cytosol, but to proteins that are tethered, either in aggregates or anchored to membranes.

We have isolated a factor, FOAD-II, from the cytosol fraction of bovine brain, which retards the rate of rundown, and sequence information has identified this as a heterodimer consisting of rac and rhoGDI. These have to be present together to have an enhancing effect on secretion: rac provided alone is without effect, while rhoGDI accelerates the rate of rundown. The simplest conclusion to draw from these observa-
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30 n
25-
20-
10-
10
GTP-γ-S, [μM]

Figure 9. Effect of FOAD-II (rac/rhoGDI complex) on the requirement for Ca²⁺ and GTP-γ-S for secretion from run-down mast cells. The cells were permeabilized and allowed to run down for 17 min in buffer A in the presence and absence of FOAD-II (5 μg ml⁻¹) before stimulation with various combinations of GTP-γ-S (abscissa) and Ca²⁺ (□ pCa5.5; ○ pCa5.25; △ pCa5). Open symbols indicate absence of FOAD and solid symbols indicate presence of FOAD. Points indicate average of three separate determinations.

The conclusion is that the rundown occurs as a consequence of the leakage of rac from the cells and that the complex of exogenous rac/rhoGDI is capable of restoring this; however, we have not shown so far that rac is indeed released from the permeabilized cells and there may be other explanations. Our conclusion is supported by the observation that recombinant rac2, preloaded with GTP-γ-S, is capable of inducing exocytosis in the absence of added guanine nucleotide (our unpublished observation).

Unfortunately we have been unable to demonstrate the leakage of rac from the permeabilized cells. Antibodies raised against rac1 and rac2 C-terminal peptides, although reactive against the recombinant forms and also a peptide running at 30 kDa (Abo et al., 1994; our unpublished observations), failed to detect the wild-type protein (24 kDa) on Western blots. Similarly, an antiprotein antibody (gift of Alan Hall) now has low sensitivity to peptides running at 24 kDa and displays considerable cross-reactivity against other proteins. Leakage of the mobile component of rhoGDI is at least as rapid as other soluble proteins such as lactate dehydrogenase (Howell and Gomperts, 1987) and phosphoglycerate kinase (Gomperts et al., 1987), and is effectively complete by 5 min. This is considerably faster than the leakage of actin that continues to emerge during 15–60 min (Koffer and Gomperts, 1989).

One possibility is that the FOAD does not act to increase the level of rac in the depleted cells, but prevents the detachment and loss of a factor, which may not be rac. Alternatively, an important factor other than rac is lost from the cells causing rundown, and provision of rac substitutes for its lost function. The GDI proteins are known to retard GDP dissociation from specific low molecular weight GTPases (Takai et al., 1992) and also to confer aqueous solubility (Isomura et al., 1991) and thus to act as escorts to the wild-type forms, all of which are otherwise insoluble due to the presence of acyl and isoprenyl attachments at the carboxyterminals (Kinsella et al., 1991; Takai et al., 1992). Guanine nucleotide exchange is enhanced only when the GTPases are transferred to their membrane binding sites (Takai et al., 1992; Bokoch et al., 1994). The essential role of the GDI gives an important clue that the first explanation, namely that rac (or a closely related GTP-binding protein) detaches and

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leaks from the permeabilized cells, and that provision of rac, in its complex with rhoGDI, is capable of reversing this loss, thus ensuring that the membrane-bound pool of rac is not exhausted. The rac content is reported as approximately 11 ng/10^6 mast cells (Price et al., 1995). For the purposes of this discussion we shall assume that it is indeed the loss of rac that causes the rundown.

The rac/rhoGDI complex, FOAD-II, is only capable of restoring activity to cells that have retained some capacity for stimulated secretion; for cells that have become fully refractory, the presence of FOAD-II is without effect. This indicates that during the initial phases of the rundown, provision of FOAD-II is able to make good whatever has been lost, which determines the limiting step in exocytosis. As the rundown proceeds and the cells become insensitive to stimulation even in the presence of FOAD-II, the loss of other proteins (possibly FOAD-I [Figure 4], and maybe others) may become critical. These may detach from their binding sites and leak through the SL-O-induced lesions at a slower rate. Note, however, that FOAD-II is still capable of causing substantial enhancement even when the unsupported level of secretion has declined to well below 5% (Figures 11 and 9).

Wild-type rac on its own has no ability to retard rundown. In contrast, recombinant, constitutively active forms of rac1 or rhoA are capable of enhancing secretion from permeabilized (and washed) mast cells (Price et al., 1995). This difference is probably due to the lack of post-translational modification of the recombinant proteins. The rhoGDI is necessary to maintain the wild-type protein in solution. Although the major effect of the rac/rhoGDI is to enhance secretion induced by GTP-γ-S, as would be expected for a function mediated by a GTP-binding protein, the complex still appears to induce secretion to a small extent in the absence of an activating guanine nucleotide (Figure 10). Although this behavior is apparently anomalous, there is a precedent for this observation in the report of activation of the NADPH oxidase complex by rac in neutrophils (Abo et al., 1994; Bromberg et al., 1994). In the search for an explanation of this paradox it may be worth recalling that the stimulation of cell surface receptors can also occur in the absence of activating ligands (Bond et al., 1995). This has been rationalized on the basis of a two-state model in which the receptors are in equilibrium between the active and the inactive conformations. The conventional agonist, rather than activating the receptor directly, has the effect of shifting the equilibrium to increase the proportion of the receptor in the active form. Similarly, we offer the idea that the ability of rac to induce a low level activation of exocytosis in the absence of GTP could be due to a two-state equilibrium in which there exists a small proportion of the rac in an active form.

The inhibitory effect of rhoGDI is best explained by a mechanism involving the extraction of rac, together with other rho-related peptides, from their native binding sites within the cells. A similar mechanism has been invoked to explain the inhibition by rhoGDI of phospholipase-D (PLD) activation in permeabilized HL-60 cells (Bowman et al., 1993). For these cells it has been suggested that the activation of PLD is central to the triggering of secretion (Stutchfield and Cockcroft, 1993) and so rhoGDI would be expected to be an inhibitor. A more direct experimental approach is needed to confirm this mechanism.

GTP is required for activation of mast cells but it appears that depending on the nature of the stimulus, separate GTP-binding proteins mediate the terminal stages of the secretory pathway. For activation by receptor-mimetic agents (mastoparan, compound 48/80, etc.), exocytosis can be inhibited by treatment with pertussis toxin, implicating a heterotrimeric G-protein as the G Protein, the GTP-binding protein mediating exocytosis (Aridor et al., 1990). Introduction of specific probes into permeabilized mast cells has identified this as G13 (Aridor et al., 1993). Conversely, secretion stimulated by cross-linking the receptors for IgE or with Ca^{2+}-ionophores is quite insensitive to ADP-ribosylating toxins (Saito et al., 1987) and yet still requires the presence of GTP (Wilson et al., 1989; Marquardt et al., 1987). Rac can therefore be considered as a candidate for the G Protein-mediating exocytosis in mast cells stimulated by activation of the IgE pathway. Alternatively, it could be the regulator of a common late event, distal to the site of action of the pertussis-sensitive step in the pathway induced by the receptor-mimetic agents, and conferring the requirement for GTP in the pathway initiated by activation of the receptor for IgE. However, the finding that exocytosis from permeabilized cells can be induced by [AlF_4]^- (Lillie and Gomperts, 1992b; Aridor et al., 1993) is indicative of a direct pathway of activation through a heterotrimeric G-protein. For this reason it is more likely that there are discrete pathways of activation for stimulation of IgE receptors or with receptor-mimetic agents.

The mechanism of action of rac in mast cells remains unclear, but there are a number of possible ways in which it may act. As discussed above PLD has been implicated in the regulation of secretion from HL-60 cells and it is possible that the rac acts directly or indirectly on this protein and in this way regulates secretion in mast cells. An alternative possibility is that the action of rac is exerted via regulation of the cytoskeleton: rac, rho, and cdc42 have all been shown to exert profound effects on the cytoskeleton (Nobes and Hall, 1995; Hall, 1994). Rac and rho proteins have been demonstrated to be involved in the disassembly of the cortical actin network in rat peritoneal mast cells stimulated by Ca^{2+} and GTP-γ-S (Norman et al., 1994).
This cortical web of actin has been proposed to regulate exocytosis in a number of secretory cells (for review see Trifaro et al., 1992) by acting as a barrier to fusion of the secretory granules and plasma membrane. However, no direct demonstration of this has yet been achieved and it remains possible that the correlation between actin disassembly and secretion is not mechanistically significant and that the effects of rac on both secretion and the cytoskeleton are due to their having a similar signaling pathway possibly through an effector such as a rac-activated kinase (Manser et al., 1994).

The importance of rac is underlined by the finding that several other cytosolic proteins, including PI-TP, 14-3-3 proteins, NSF, α-SNAP, γ-SNAP, calmodulin, and annexin-II (both the heterotetramer and the monomeric forms), all known to modulate exocytosis in other secretory systems (Ali and Burgoyne, 1990; Morgan and Burgoyne, 1992; Nishizaki et al., 1992; Hay and Martin, 1993; Hay et al., 1995; Morgan et al., 1993; Roth et al., 1993; Bond et al., 1995), are without effect in the mast cell under the conditions of our experiments. It is likely that their leakage from these cells following permeabilization by SL-O is substantial and probably rapid, but although their loss is apparently not rate limiting, this does not mean that they are without a role. In contrast, the function supported by the FOAD-II complex of rac and rhoGDI is clearly of considerable importance.

ACKNOWLEDGMENTS

This work has been financed by a Programme Grant from the Wellcome Trust, with additional support from the Vanderfoull Foundation and the Gower Street Secretory Mechanisms Group. We thank Alan Morgan and Robert Burgoyne for preparations of 14-3-3 proteins (Exo1), NSF, α, and γ-SNAP, Geraint Thomas for a preparation of phosphatidylinositol transfer protein, and Louise Upton for help with the preparation of annexin-II.

REFERENCES


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(i.e. after patch rupture) (Oberhauser et al., 1992). Under such conditions, thorough dialysis of the cell interior through the single lesion will have taken place (Penner et al., 1987). The persistence of the exocytotic response in rat mast cells in the whole cell configuration provides an opportunity to test the ability of specific proteins to inhibit membrane fusion. Since GTP binding proteins of the Rho family have been implicated in exocytosis (Price et al., 1995; O'Sullivan et al., 1996), we have investigated the effect of RhoGDI by introducing it into rat mast cells through a patch pipette, prior to stimulation with GTP-γ-S, monitoring changes in membrane capacitance as a measure of exocytosis.

Results

Effect of native RhoGDI

We have tested the effect of both the native and recombinant forms of RhoGDI on GTP-γ-S-induced exocytosis in rat mast cells, by including these proteins in the patch pipette solution. To allow time for exogenous proteins to enter the cell and to locate before stimulation, caged GTP-γ-S (guanosine-5′-O-(3-thiotriphosphate), 3′-S-[1-(4,5-dimethoxy-2-nitrophenyl)ethyl]thio ester) was provided in the pipette solution. Cells were then maintained in the whole cell configuration for either 5 or 10 min before brief irradiation with UV light to release the active, non-hydrolysable nucleotide. When no proteins were present in the patch pipette, exocytosis, measured as an increase in cell capacitance (∆Cm), was always complete within ~10 min of photolysis (completion time = 511 ± 80 s; ∆Cm = 13.2 ± 1.0 pF; mean ± SE, 12 cells). An example is shown in Figure 1A.

RhoGDI protein was isolated from bovine brain as described previously (O'Sullivan et al., 1996). Purification was achieved by a series of chromatographic steps using the run-down bioassay. The final preparation gave a single band, visualized by silver staining, on analysis by SDS-polyacrylamide gel electrophoresis (O'Sullivan et al., 1996). When the purified protein was included in the pipette solution, inhibitory effects were detected when the light pulse was given at either 5 or 10 min after patch rupture. In 6 out of 7 cells stimulated at 5 min, exocytosis was completely inhibited by 10 μg/ml purified RhoGDI. A typical record is shown in Figure 1A. When, under the same conditions, RhoGDI was provided at only 1 μg/ml, it failed to prevent exocytosis in any of the 8 cells studied.

Fig. 1. Exocytosis in mast cells induced by photolysis of caged GTP-γ-S and its inhibition by RhoGDI purified from brain. Mast cells were maintained in the whole cell configuration for either 5 or 10 min, at which time they were exposed to UV illumination for 1 min. The pipette solution (see Materials and methods) included caged GTP-γ-S. (A) Examples of relative capacitance changes following stimulation at 5 min. The pipette solution contained no protein or included native RhoGDI at 1 or 10 μg/ml. (B) The effect of photoreleasing GTP-γ-S within the patch pipette before patch rupture (left-hand trace) and 10 min after patch rupture when native RhoGDI (5–10 μg/ml) was included in the pipette solution. The effect of heat-denatured protein (20 μg/ml, ‘Boiled’) upon exocytosis triggered at 10 min is also shown. (C) A portion of a capacitance record (cell loaded with 1 μg/ml RhoGDI), plotted at high resolution to show the stepwise increase in capacitance. A simultaneous record of access conductance is shown below.

RhoGDI (20 μg/ml) did not inhibit exocytosis (Figures 1B and 3A).

Alternatively, when 5–10 μg/ml RhoGDI was present in the pipette solution, but the pulse of UV light was applied just before patch rupture (in the cell-attached configuration), secretion commenced immediately. This is illustrated by the trace on the left in Figure 1B. GTP-γ-S was released within the pipette and, since it diffuses rapidly (Pusch and Neher, 1988), it initiated exocytosis before appreciable amounts of protein had entered the cell. This confirms that the cells were secretion competent before protein entry, that the light pulse did not damage them and that the inhibition observed was not caused by any rapidly diffusing substance in the pipette solution.

Confirmation that the observed capacitance increases were...
Rho guanine nucleotide dissociation inhibitor protein (RhoGDI) inhibits exocytosis in mast cells

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Introducing non-hydrolysable analogues of GTP into the cytosolic compartment of mast cells results in exocytotic secretion through the activation of GTP binding proteins. The identity and mechanism of action of these proteins are not established. We have investigated the effects of Rho GDP dissociation inhibitor (RhoGDI) on exocytosis induced by guanosine 5′-O-(3-thiotriphosphate) (GTP-γ-S) in rat mast cells, introducing the protein into cells by means of a patch pipette and recording the progress of exocytosis by monitoring cell capacitance. To allow time for the protein to enter the cells and find its correct location, stimulation was provided 5–10 min after patch rupture by photolysing caged GTP-γ-S included in the pipette solution. When bovine RhoGDI was introduced into mast cells, exocytosis was inhibited at concentrations of 200–400 nM for native protein and 800 nM to 8 μM for the recombinant form. Protein denatured by heat or N-ethylmaleimide treatment did not inhibit. In permeabilized cells, recombinant RhoGDI increased the rate at which cells lose their ability to respond to GTP-γ-S. These data demonstrate that one or more small GTP binding proteins of the Rho family has a central role in the exocytotic mechanism in mast cells. Keywords: exocytosis/GTP binding proteins/patch–clamp capacitance

Introduction

Non-hydrolysable analogues of GTP can activate exocytosis when delivered directly to the cytoplasmic compartment of rat peritoneal mast cells (Lillie and Gomperts, 1992) and guinea pig eosinophils (Nüsse et al., 1990) either by membrane permeabilization or through a patch pipette. Under these conditions, there is neither an absolute requirement for the elevation of Ca²⁺, nor is the presence of ATP essential at the time of stimulation. This is in contrast to other types of regulated secretory cells, such as neurons and many hormone-secreting cells, in which Ca²⁺ is an essential trigger of exocytosis. The mechanism of action and the identity of the GTP binding proteins that are involved in the final steps of the secretory mechanism in mast cells and eosinophils are not yet established, although some evidence exists for the involvement of Gx₁₃ (Aridor et al., 1993).

Rat peritoneal mast cells permeabilized by streptolysin O (SL-O) in the presence of guanosine 5′-O-(3-thiotriphosphate) (GTP-γ-S) and Ca²⁺ buffered in the micromolar range, can release approaching 100% of their stored products (Howell et al., 1987). However, if this stimulus is provided subsequent to permeabilization, the response is reduced, declining progressively as the cells lose cytosol proteins (Howell et al., 1989). This 'run-down' of secretion has been used as the basis of an assay to test the ability of factors isolated from brain cytosol to support secretion (O’Sullivan et al., 1996). One such factor was identified as the protein Rac1, a GTP binding protein of the Rho family. Rac was shown to support secretion by retarding the run-down of SL-O-permeabilized mast cells, but only when it was provided as a complex with RhoGDI (Rho GDP dissociation inhibitor). In similar experiments, purified RhoGDI provided alone accelerated the run-down of the secretory response after permeabilization (O’Sullivan et al., 1996). RhoGDI interacts with all members of the Rho family and can inhibit GDP release from, and GTP-γ-S binding to, post-translationally processed Rac1 and Rac2 (Ando et al., 1992).

Exocytosis is characterized by the fusion of secretory granule membranes with the cell plasma membrane. Measuring the release of secretory products from permeabilized cells provides, at best, an indirect means of assessing its progress. There is also the risk that, under some conditions, damaged or leaky granules might give rise to release that is not exocytotic. Additionally, permeabilizing agents such as SL-O and digitonin may themselves interfere with the protein and membrane interactions that mediate and characterize exocytosis. A direct method of monitoring exocytosis that provides intracellular access but requires no permeabilizing agent is provided by the whole cell patch–clamp capacitance technique. The progress of exocytosis is followed by monitoring the increase in cell capacitance that occurs as secretory granules fuse with the plasma membrane (Lindau and Neher, 1988; Lindau, 1991). This method also has the advantage that, in contrast to permeabilized cells, the disruption to the plasma membrane caused by a patch pipette is localized to a small region. [For example, a pipette with an outer diameter as large as 4 μm in contact with a mast cell of diameter 13 μm (Helander and Bloom, 1974) will perturb <5% of the total surface.]

When GTP-γ-S is provided through a patch pipette in the whole cell configuration, rat mast cells undergo complete exocytosis, in common with permeabilized cells, and measurements of membrane capacitance (and conductance) have provided evidence of the individual fusion events showing that the release is truly exocytic (Fernandez et al., 1984). However, in contrast to the permeabilized cells, the response of patched clamped rat mast cells does not decline when the stimulus is provided 15–20 min after achieving the whole cell configuration.
due to exocytosis is provided by examining the capacitance records at high resolution. Part of a record from cells loaded with 1 μg/ml RhoGDI is reproduced in Figure 1C using an expanded scale, revealing the sequence of capacitance steps that indicate individual secretory granules fusing with the plasma membrane. These changes in capacitance are not accompanied by any significant changes in access conductance.

**Effects of recombinant RhoGDI**

Recombinant bovine RhoGDI-GST fusion protein (rRhoGDI) was expressed in transformed *Escherichia coli* (Hancock and Hall, 1993) and purified. Analysis by SDS-PAGE revealed a single band (Figure 2). Introduction of rRhoGDI into cells revealed inhibitory effects that were not as marked as those of the native protein. Mast cells were loaded with either rRhoGDI, boiled rRhoGDI or N-ethylmaleimide (NEM)-treated rRhoGDI and stimulated by photolysis of caged GTP-γ-S as described above. While exocytosis was completely inhibited in some cells, in others reduced or slowed responses were observed. The data summarized in Figures 3 and 4A reflect these three phenomena. In Figure 3A, inhibition manifest as the complete absence of exocytosis is depicted by showing the proportion of cells that responded with an increase in capacitance, however small. In Figures 3B and C and 4A, the characteristics of exocytosis in the responding cells, i.e. those in which exocytosis had not been completely suppressed, are summarized. Figure 3B shows the extents of the capacitance increases and Figure 3C shows the times taken (after stimulation) to reach completion. The inhibitory effect of the native protein (5–10 μg/ml) is much greater than that of the recombinant protein at higher concentrations (Figure 3A). At 100 μg/ml rRhoGDI, exocytosis was prevented in only two out of nine cells. However, the extent of secretion of the seven responding cells was about a half of that of the controls (Figure 3B) and the completion time was 80% longer (Figure 3C). At 200 μg/ml, exocytosis was prevented in 4 out of 7 cells and the secretion time of the three that underwent exocytosis was extended by 74%.

The control experiments are also summarized in Figure 3 (hatched bars). When the recombinant protein had been boiled, it failed to inhibit or slow secretion when included at up to 200 μg/ml in the pipette solution. NEM-treated RhoGDI (200 μg/ml) also failed either to reduce or slow secretion.

When rRhoGDI was provided at 20 μg/ml, only one cell out of 10 failed to secrete. However, the progress of exocytosis in the 9 responding cells was quite different from that of the controls. The extent of secretion (Figure 3B) was reduced and, although the time to completion was not different from the controls, the rate of exocytosis was lower especially in the early stages. This is illustrated in Figure 4A where the derivative of averaged capacitance traces during the 200 s following stimulation is shown.

**Effect of rRhoGDI on capacitance steps**

The slowing effect of rRhoGDI on the progress of exocytosis is also demonstrated by analysis of the step increases in capacitance that characterize individual granule fusions. The distribution of step sizes for cells loaded with rRhoGDI and boiled rRhoGDI do not differ

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**Fig. 2. Purity of recombinant RhoGDI-GST fusion protein. rRhoGDI was prepared as described in Materials and methods and analysed by SDS-PAGE by extraction into standard Laemmli sample buffer (Laemmli, 1970) under denaturing conditions and separation on a 12% gel. Visualization was by silver staining. The protein load was 1.6 μg. The figures indicate relative molecular mass in kDa.**

**Fig. 3. Inhibition of exocytosis by both native and recombinant RhoGDI. These data summarize the inhibition of exocytosis in three ways. In (A) complete suppression of exocytosis is recorded by showing the proportion of cells that responded with any increase in capacitance. The figures above each column indicate the number of cells examined in each category. (B) and (C) describe the responses of cells in which exocytosis was not completely suppressed. (B) summarizes the extents of the capacitance increases and (C) the time taken to complete exocytosis after stimulation of the responding cells. Data in (B) and (C) are mean ± SE. Numbers of cells are indicated above each column in (B). Significant differences between each set of data and the boiled control are indicated by asterisks. * indicates P < 0.05 and ** indicates P < 0.01 (Mann-Whitney U test).**
Inhibition of exocytosis by RhoGDI

**Table I.** The time constant for cells loaded with rRhoGDI

time constant was calculated. The results are shown in

for cells loaded with rRhoGDI at 100 µg/ml it was longer
by a factor of 2.

*rRhoGDI accelerates run-down in permeabilized cells*

An indication that RhoGDI might be inhibitory to
exocytosis was first obtained in experiments with partially
responsive mast cells permeabilized by SL-O (O’Sullivan
*et al.*, 1996). Acceleration of run-down by the purified
protein was observed, beginning as early as 5 min after
permeabilization, but the extent of this effect was difficult
to assess. This is because the rate at which permeabilized
cells run down varies widely from batch to batch and
because of the steepness of the run-down curves. Because
of its variability and in view of the concern that the run-
down phenomenon might be an artefact of the permeabiliz-
ing agent SL-O, we have compared the effects of rRhoGDI
in patched and SL-O-permeabilized cells. Figure SA shows
the effect of different protein preparations upon mast cells
that were permeabilized at time zero and challenged at
subsequent times with GTP-γ-S (100 µM) and Ca²⁺
(pCa 5). In the presence of rRhoGDI, the run-down of
the response is more rapid. The concentration dependence
of the effect on run-down is shown in Figure 5B. The
relative cell responsiveness is expressed as the ratio of
secretion stimulated by GTP-γ-S 10 min after permeabiliz-
ation in the presence of boiled rRhoGDI to that with intact
protein. The EC₅₀ for the acceleration of run-down is in
the region of 7 µg/ml. At 20 µg/ml, the concentration at
which the effects of the recombinant protein become
apparent in patch-clamped cells, the relative responsive-
ness of the permeabilized cells is at its lowest.

**Discussion**

To test the effect of an exogenous protein on the mechanism
of exocytosis requires its introduction into the cell by a
method that does not compromise its ability to undergo
membrane fusion. To perfuse the cell interior effectively
with a macromolecular solute requires the creation of
substantial holes in the plasma membrane. These may be
created by permeabilization or by a patch pipette. Because
macromolecules diffuse slowly, several minutes may be
required for them to accumulate within the cell. During
this period, leakage of both low molecular mass solutes
and cytosolic proteins occurs from the cell and, in con-
sequence, its ability to respond to a stimulus may diminish
with time. For example, rat mast cells permeabilized by
SL-0 gradually lose their ability to respond to GTP-γ-S
over a 20 min period (Howell *et al.*, 1989) (Figure 5A).
Patch-clamped rat mast cells, however, do not run down
appreciably in the 20 min following patch rupture. The
requirements of the patch-clamp technique impose working
conditions quite different from those of the permeabilized
cell experiments and these may account
for the difference in responsiveness. For example, the intracellular solution has different ionic composition and
pH (see Materials and methods). Also the electrophysio-
logical experiments were performed at room temperature
rather than at 30°C. In addition, the patch-clamped cells
were attached to glass coverslips and this may have
activated adhesion molecules that interact with intracellu-
lar systems such as the cytoskeleton. Finally, the lesions

![Fig. 4. Effect of rRhoGDI on the progress of exocytosis in responding cells. In (A) the rate of exocytosis (the derivative of averaged
capacity records) is plotted against time for cells loaded with either
20 µg/ml (10 cells) or 100 µg/ml (9 cells) rRhoGDI, or with
200 µg/ml NEM-treated rRhoGDI (9 cells) or 200 µg/ml boiled
rRhoGDI (5 cells). (B) The effect of rRhoGDI upon the timing of
capacitance steps. Histograms of the interval between successive steps
during the first 30% of each capacitance increase are shown. The
curves are single exponential fits (see Materials and methods). For
rRhoGDI at 100 µg/ml the time constant of the fitted curve is
200 ms; for boiled RhoGDI the time constant is 674 ms. Further
details are given in Table I.](image-url)
generated by SL-O are distributed over the whole plasma membrane in contrast to a single hole made by the patch pipette, possibly allowing more leakage from the permeabilized cells. All in all it is clear that there are substantial differences between these two experimental systems. [Note, incidentally, that patch-clamped mast cells from mutant beige mice do run-down, i.e. their ability to respond to (photoreleased) GTP-γ-S is lost within ~5 min; P.Mariot and P.E.R.Tatham, unpublished.] We have taken advantage of the persistence of the exocytotic response in rat mast cells to enable us to load them with exogenous protein, in particular RhoGDI, through a patch pipette, detecting exocytosis by monitoring capacitance. The effect of the loaded protein upon exocytosis was tested by applying a stimulus (photoreleased GTP-γ-S) at up to 10 min after breaking into the cell. In control experiments, the pipette solution contained either inactivated proteins or no protein at all, and uncaging GTP-γ-S resulted in large, rapid capacitance increases, characteristic of full exocytosis. This confirmed that the exocytotic machinery remained intact throughout the loading period. Full exocytosis also occurred promptly when inhibitory protein was not given sufficient time to diffuse into the cell (Figure 1B).

The introduction of bovine RhoGDI, either purified from brain or a recombinant form, has an inhibitory effect upon exocytosis in mast cells. RhoGDI purified from brain is a potent inhibitor (Figure 3A), almost completely suppressing exocytosis at 5–10 µg/ml or 200–400 nM (calculated M₀ = 23 421; Fukumoto et al., 1990). The inhibitory effects of this protein are detectable as early as 5 min after patch rupture (Figure 1A). rRhoGDI is also inhibitory, but it is not as effective as the native protein even when provided at a 20-fold higher concentration (up to 200 µg/ml or 8 µM, Figure 3A). The lower efficacy of the recombinant protein compared with its native form may be due to the fact that one or more domains are incorrectly folded. Alternatively, it may lack a post-translational modification, such as a phosphorylation, that is required for its full activity. It has been reported that dephosphorylation of purified RhoGDI reduces the stability of the Rho A–RhoGDI complex (Bourmeyster and Vignais, 1996).

Following previous work, in which native RhoGDI was shown to accelerate run-down in permeabilized mast cells (O'Sullivan et al., 1996), we now show that recombinant RhoGDI has a similar effect and demonstrate its concentration dependence (Figure 5). Detailed concentration–response data are difficult to obtain from patch-clamp experiments. The EC₅₀ for inhibition is 7 µg/ml or ~300 nM (Figure 5B). The lowest concentration at which we have observed consistent effects of this protein in patch-clamped cells is 20 µg/ml. Bearing in mind the considerable dissimilarities between the two experimental systems, these values are comparable and we conclude that the permeabilized cell data provide additional evidence of inhibition by the recombinant protein.

When the capacitance records are examined in more detail to reveal the individual granule fusions that comprise exocytosis, it becomes clear that the slowing of secretion caused by rRhoGDI is due to extended intervals between successive capacitance steps (Figure 4B and Table I). The
Cdc42 proteins are either attached to membranes where they act as appropriate. Percoll steps as described previously (Tatham and Gomperts, 1990) and Dawley rats (300–400 g). For patch-clamp studies, mast cells were washed to remove unbound SL-0 by centrifugation and resuspension at ice temperature. Membrane capacitance was monitored by the patch-clamp technique using the whole cell configuration and a digital phase detector (Joshi and Fernandez, 1988; Monck et al., 1990; Fidler and Fernandez, 1989). A sinusoidal voltage (830 Hz, 50 mV peak–peak) was applied to the command input of an EPC-7 patch–clamp amplifier at a holding potential of 0 mV. Current amplitude was recorded at two orthogonal phase angles to provide signals proportional to the real and imaginary parts of the admittance. The phase alignment was determined periodically by the phase tracking technique (Fidler and Fernandez, 1996). The real and imaginary components reflected changes in access resistance and capacitance respectively. The pipette solution contained 140 mM potassium glutamate, 10 mM HEPES, 7 mM MgCl₂ and 50 μM ATP Mg buffered to pH 7.2 with KOH. K. EGTA and Ca. EGTA were also present to give a total EGTA concentration of 10 mM and pCa 6.7 to pCa 6.3. To stimulate exocytosis, 110 μM caged GTP-γ-S was included in this solution. Pipettes were coated with Silgard and had resistances of 1–3 MΩ. The mean initial access conductance was 114 ± 3 nS and the mean final access conductance was 84 ± 4.5 nS (SEM, n = 60). The cell membrane resistance (d.c. current) did not change appreciably during capacitance measurements. When proteins were present in the intracellular solution, the tip of the pipette was dipped in protein-free solution for a few seconds before back-filling. For calculations of the extents of the capacitance increases and of the completion times of exocytosis, the end-point was taken as the appearance of a plateau in the capacitance trace or the achievement of a level within 95% of the maximal change. All patch–clamp experiments were carried out at room temperature (20–25°C). Histograms of capacitance step intervals were constructed from data obtained from the initial 30% of the capacitance increase for each cell analysed. Curve fitting to these histograms was performed using ‘Origin’ (Microcal Software Inc.). Single exponential fits, yielding values of time constant, χ² and SE, were obtained by the Levenberg–Marquardt method, weighting each data value statistically (1/σ).

Materials and methods

Cells

Rat mast cells were prepared by peritoneal lavage of male Sprague–Dawley rats (300–400 g). For patch–clamp studies, the cells were washed once in a buffer containing 137 mM NaCl, 2.7 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂, 10 mM HEPES pH 7.2 and placed on glass coverslips mounted in plastic Petri dishes. For permeabilized cell experiments, mast cells were purified to >99% purity by centrifugation through a Percoll step as described previously (Tatham and Gomperts, 1990) and suspended in buffer containing 137 mM NaCl, 2.7 mM KCl, 1 mM MgCl₂, 20 mM HEPES pH 6.8 and 3 mM Ca. EGTA to maintain pCa 5 or pCa 8 as appropriate.

Membrane proteins were heat-denatured by maintaining them at 95°C for 15 min. Reconstituted RhoGDI was also inactivated by exposure to NEM (1 mM, 6481
37°C, 30 min). The residual NEM was then neutralized by incubation with diithiothreitol (DTT, 2 mM, 30 min) and the modified protein was passed through a gel filtration column (NAP5) to remove DTT. The final preparation was homogeneous as assessed by anion-exchange chromatography, gel filtration and SDS–PAGE. Inactivation of RhodGDI was tested by using it to extract GTP binding Rho-related proteins from permeabilized mast cells. Briefly, mast cells were permeabilized by SL-O in the presence of RhodGDI that had been pre-treated with (or without) NEM. After 25 min, the cells were centrifuged (14000 × g) and an acetonate precipitate of the supernate was prepared. The precipitate was dispersed in standard sample buffer (Laemmli, 1970), subjected to SDS–PAGE, blotted, on to nitrocellulose and overlayed with [α-32P]GTP. Autoradiography of the blot then revealed the GTP binding proteins extracted from the cells by both NEM-treated and unmodified RhodGDI. The level of GTP binding obtained using NEM-treated RhodGDI was 28% of the control.

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